EFFECTS OF THE COMPONENTS OF THE GET PATHWAY ON PRION PROPAGATION

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EFFECTS OF THE COMPONENTS OF THE GET PATHWAY ON PRION PROPAGATION

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I dedicate this work to my dad Prof. (Dr.) Lalit Mohan Bariar, my mom Mrs. Deepali Bariar, my husband Dr. Nilabh Srivastava, and my younger brother Vineet Bariar. This work would not have been possible without their perpetual love, faith, support, and encouragement.

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CHAPTER 1

INTRODUCTION

Prions

One of the most important discoveries of modern molecular biology is that information can be transmitted not only by nucleic acids, but also by infectious proteins called Prions. Prions are self-perpetuating protein isoforms that propagate by converting the normal form of the protein of the same amino acid sequence into the infectious form (27). Prions cause fatal neurodegenerative diseases in mammals e.g. Bovine Spongiform Encephalopathy (Mad Cow disease) and Creutzfeldt-Jacob disease in humans (1). Mammalian prions form fiber-like aggregates rich in β-sheet structures known as amyloid fibers. Amyloid fibers are associated with neurodegenerative diseases such as Alzheimer's disease (AD), Huntington's disease (HD) etc.

Prions control heritable traits in yeast. [*PSI*$^{+}$] is a self-polymerized isoform of the yeast *Saccharomyces cerevisiae* eukaryotic release factor (translation termination factor eRF3) Sup35. During translation the soluble (non-prion) form of [*PSI*$^{+}$] along with Sup45 recognizes stop codons. An altered protein conformation of Sup35 creates the [*PSI*$^{+}$] prion. [*PSI*$^{+}$] alters translational fidelity and creates a nonsense suppressor phenotype (2) and allows the growth on medium lacking adenine in strains containing the *ade1-14* reporter. (Fig. 1.1B). On the other hand, in cells containing soluble Sup35 protein (Fig 1.1A), there is no growth on medium lacking adenine due the efficient termination at stop codon of *ade1-14* reporter.

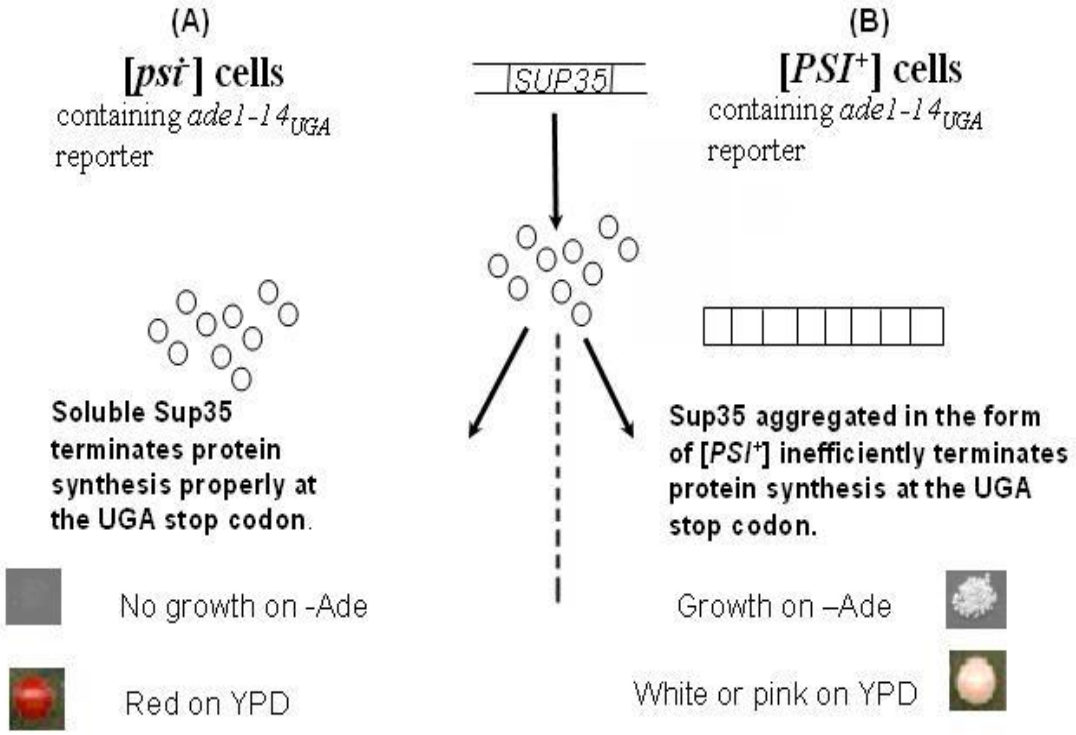

Figure 1.1 **Nonsense Suppressor Assay for the [*PSI*$^+$] prion**
The *ade1-14$_{UGA}$* reporter system is used to detect the presence of [*PSI*$^+$] prion. As shown in **A**, in [*psi*$^-$] cells the Sup35 protein is in its soluble form and efficiently terminates protein synthesis at the UGA stop codon in the *ade1-14$_{UGA}$* mRNA. In this case enough Ade1 product is not formed causing lack of growth on –Ade media and red color on YPD. On the other hand when the Sup35 protein is aggregated to form the [*PSI*$^+$] prion (**B**), the translation termination function is impaired and it does not efficiently recognize the stop codon. This leads to enough production of Ade1 which enables the cells to grow on –Ade media. The color in this case is white or pink on YPD

There are three broad regions in the Sup35 protein structure- the N-terminal region which is the prion domain which is not required for the normal cellular function of the protein, but required for prion formation and propagation. It is QN-rich and contains multiple imperfect repeats. Then there is the middle M region which is rich in charged residues and has some role in [*PSI*$^+$] stabilization. The C-terminal region is required for the cellular function of the protein but not for the [*PSI*$^+$] phenotype (3) (Fig 1.2 A).

There are other prions in yeast e.g. [URE3] and [*PIN*$^+$]/ [*RNQ*$^+$]. Yeast prion proteins have prion domains that are responsible for prion formation. These domains are not required for the normal function of the protein within the cell. [*PIN*$^+$] is the prion form of the protein Rnq1 (Fig. 1.2B). The function of this protein is not known. Its prion

domain is towards the C-terminus and is QN-rich. In the presence of [*PIN*$^+$] prion, overexpression of Sup35 can induce de novo [*PSI*$^+$] (4).

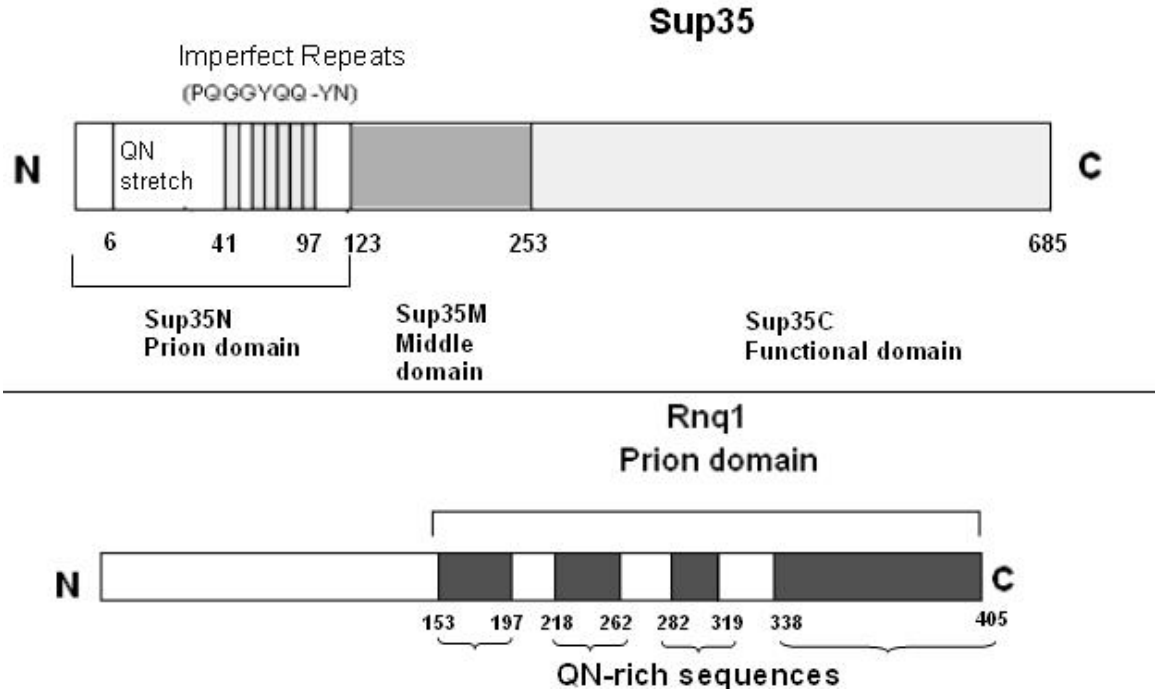

Figure 1.2 **Structural organization of Sup35 and Rnq1**
A. There are three structural domains in Sup35. There are a total of 685 amino acids in this protein. The N-terminal region (Sup35N) is the prion domain required for prion formation and propagation. It is rich in Gln (Q), Asn (N), Gly, and Pro residues and contains multiple imperfect repeats. The end of N domain is not clearly known. It is generally placed between amino acid positions 113 and 137. In this figure the end of the N domain is shown at position 123. The middle domain (Sup35M) is rich in charged residues and has some role in [*PSI*$^+$] stabilization. The C-terminal region (Sup35C) is required for the normal cellular function of the protein. **B.** Rnq1 protein has the prion domain towards the C-terminus which comprises of QN-rich sequences. The function of this protein is not known. Its prion form is [*PIN*$^+$].

Heat Shock Proteins

Heat shock proteins (Hsp's) act like molecular chaperones ensuring that the cell's proteins are folded properly. They are induced when a cell undergoes various types of environmental stresses like heat, cold and oxygen deprivation. There are different families of the cytoplasmic molecular chaperones in yeast e.g. Hsp100, Hsp70 and Hsp40. Proteins which are damaged under conditions of stress are disaggregated and properly refolded by the action of these chaperone proteins (5). Propagation of yeast prions requires components of this cellular stress response system. Hsp104 is the first

cellular protein shown to affect [*PSI*$^{+}$] propagation (8). It is a member of the Hsp100 family. It acts as a homohexamer and is an ATPase. It acts on aggregated stress-damaged proteins and disaggregates them (5). It is thus responsible for induced thermotolerance and response to other environmental stresses in yeast (Fig 1.3A). Induced thermotolerance is the phenomenon where cells acquire tolerance to heat when given a mild pre-heat treatment during which Hsp104 is induced (6). Basal thermotolerance measures the basal activity of Hsp104 through cell viability after a heat shock without any pre-heat treatment. Moderate levels of Hsp104 are required to break the [*PSI*$^{+}$] aggregates into smaller seeds which initiate new rounds of propagation (7) (Fig. 1.3B).

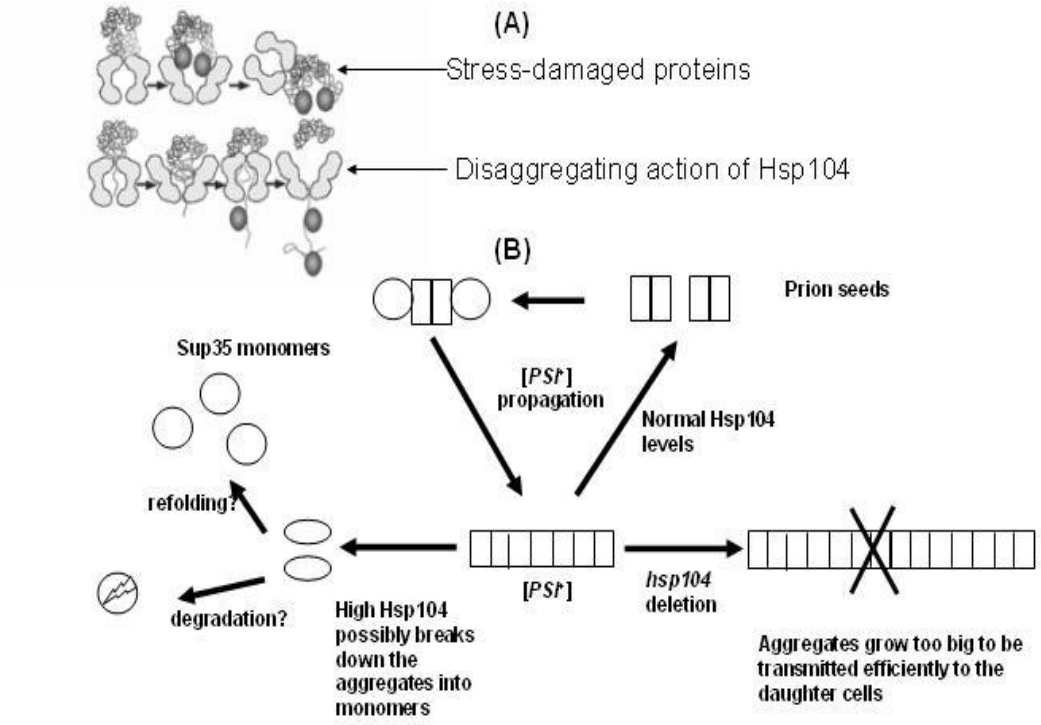

Figure 1.3 **The normal cellular function of Hsp104 and its role in [*PSI*$^{+}$] maintenance and propagation A.** The normal cellular function of Hsp104 is disaggregation of stress-damaged proteins. As a result of this ability, it produces thermotolerance in the cells (discussed in chapter 5). Figure taken from http://biochemistry.utoronto.ca/glover/lab/research.html **B.** Moderate levels of the Hsp104 chaperone are required for [*PSI*$^{+}$] propagation through the production of seeds which initiate new rounds of propagation, whereas it has been hypothesized that Hsp104 overproduction cures yeast cells of [*PSI*$^{+}$] by breaking down the aggregates into monomers. In the case of *hsp104* deletion, the aggregates become too big to be efficiently passed on to the daughter cells which are then cured of the prion.

One model suggests that excess Hsp104 cures the cells of [*PSI*$^+$] by possibly breaking it down into monomers (8). Alternatively it has been proposed that overexpression of Hsp104 may impair segregation of prions in cell divisions (36). In the absence of Hsp104 aggregates grow too big to be efficiently transmitted to daughter cells. Hsp104 also plays a role in the propagation of the other prions [*PIN*$^+$] and [URE3]. While an Hsp104 deletion cures the cell of all the three prions: [*PSI*$^+$], [*PIN*$^+$] and [URE3]; over expression of Hsp104 only cures the cell of [*PSI*$^+$]. The other two prions are stable in the presence of excess Hsp104 (4, 9) (Table 1.1).

Table 1.1 **The role of Hsp104 in yeast prion propagation**

Gene	**Protein**	**Function**	**Prion**	**Excess Hsp104**	***hsp104*D**
SUP35	Sup35	Translation termination	[*PSI*$^+$]	cures	Cures
RNQ1	Rnq1	Unknown	[*RNQ*$^+$]/ [*PIN*$^+$]	stable	Cures
URE2	Ure2	Nitrogen catabolism	[URE3]	stable	Cures

A study was carried out in this laboratory aiming at the identification of additional factors that cause this difference in curing of prions by excess Hsp104 and are responsible for the curing of [*PSI*$^+$] by over expression of this chaperone (S. Muller, J. Patterson and Y. Chernoff, unpublished data; J. Patterson Honors Thesis [23]). EMS mutagenesis was performed to generate mutants with decreased [*PSI*$^+$] curing by Hsp104 over expression. 13 mutants were phenotypically characterized and library screen was performed to identify the mutated gene in one of the mutants with the strongest phenotype. This mutant was found to have a point mutation in the *GET2* gene resulting in a premature stop codon at position 473, which leads to the formation of a truncated protein lacking the transmembrane domains. This is referred to as mutant *get2-473* (Fig. 1.4A). Get2 (Rmd7) is a component of the Get Complex which is required for the retrieval of HDEL proteins from the Golgi to the Endoplasmic reticulum (ER) (retrograde

vesicle-mediated transport) in an Erd2 dependent fashion (10) (Fig 1.4B). The other two components of the Get complex are Get1 (Mdm39) and Get3 (Arr4).

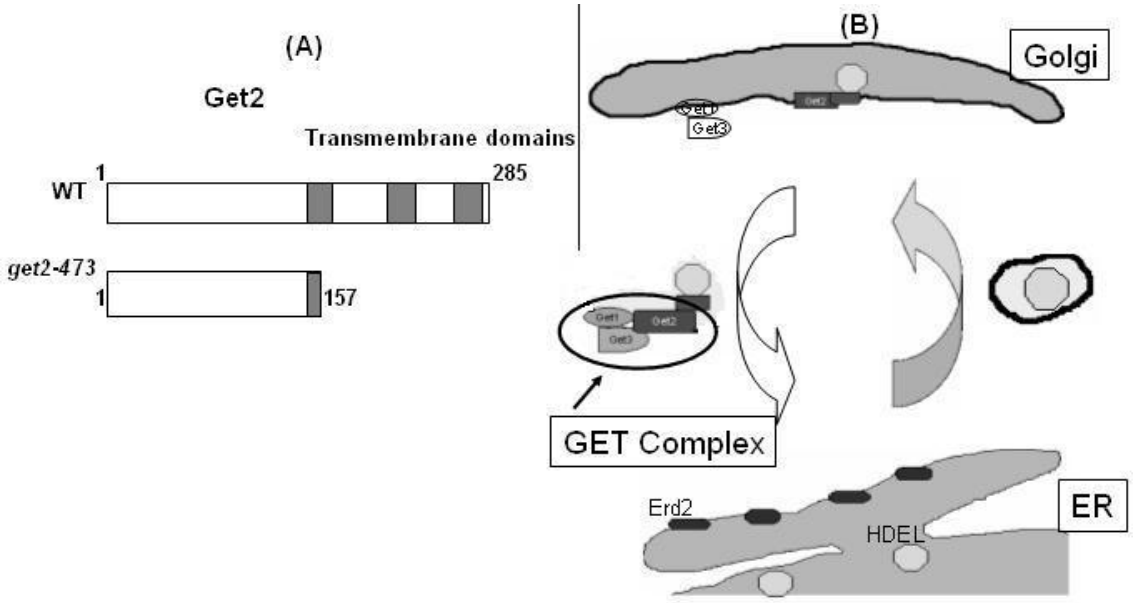

Figure 1.4 **Function of Get complex and the effect of the *get2-473* mutation on the structure of Get2 protein**
A. A truncated Get2 protein is formed in ***get2-473*** mutant due to the generation of a premature stop codon at position 473. The truncated protein lacks transmembrane domains since it has only 157 amino acids as compared to 285 in wild type. Transmembrane domains may be necessary for the correct localization of Get2 and possibly for its proper function. **B.** Get2 (Rmd7) is a component of the Get Complex which is required for the retrieval of HDEL proteins from the Golgi to the Endoplasmic reticulum (ER) (retrograde vesicle-mediated transport) in an Erd2 dependent fashion (10). The other two components of the Get complex are Get1 (Mdm39) and Get3 (Arr4). The schematic is shown here with modifications from (10).

A deletion was made in the *GET2* gene in the strong [*PSI*$^+$] strain OT56 (Table 2.1). Strong [*PSI*$^+$] strains usually have a strong nonsense suppression phenotype compared to weak strains. Also, the aggregates are smaller in size in the strong strains which leads to efficient propagation (2). The *get2Δ* strain exhibits a [*PSI*$^+$] curing defect in the presence of excess Hsp104 as well as spontaneous loss of [*PSI*$^+$] prion when grown on synthetic medium, indicated by the spontaneous appearance of red and pink colonies (S. Muller, J. Patterson and Y. Chernoff, unpublished data, and 23) (Fig. 1.5).

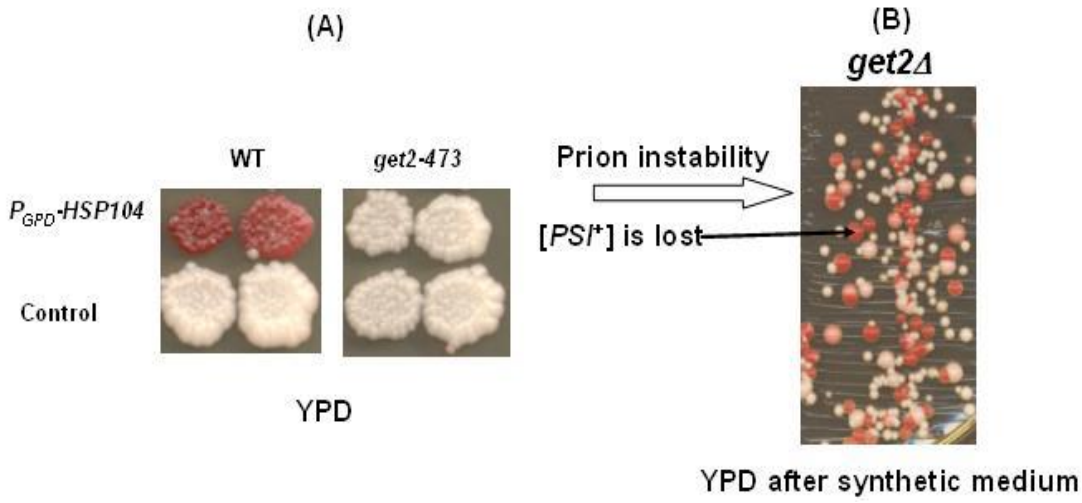

Figure 1.5 **Effect of *get2* on [*PSI*$^+$] curing and stability**
A. The wild type strain is efficiently cured of [*PSI*$^+$] in the presence of pLH105 (P_{GPD}-*HSP104)* and appears red on YPD. On the other hand *get2-473* shows a [*PSI*$^+$] curing defect by excess Hsp104 produced by pLH105 as seen by white color on YPD (S. Muller, J. Patterson, and Y. Chernoff, unpublished data; and 23).
B. Growth of *get2Δ* strain on synthetic medium causes [*PSI*$^+$] instability. The strain is streaked out on YPD after growth on complete synthetic medium (~3-4 days) and the instability can be seen by the appearance of many [*psi*$^-$] colonies which become red on YPD.

The Get Complex

The Get (Golgi-to-ER traffic) complex is required for the ATP-dependent retrieval of HDEL motif-containing proteins from the Golgi to the Endoplasmic reticulum (retrograde vesicle-mediated transport) in an Erd2 dependent fashion (10) (Fig. 1.4B). This complex comprises of three proteins namely, Get1, also known as Mdm39; Get2, also known as Rmd7; and Get3, also known as Arr4. These three components of the Get complex show physical and genetic interactions with each other (10, 11). These proteins also have some additional functions. Get1 is also required for normal mitochondrial morphology and inheritance (12). Get2 was originally discovered in a large-scale screen for genes that are necessary for meiosis (13). It is involved in cell wall organization and biogenesis and might be involved in cell wall function. *get2Δ* exhibits defects in growth on non-fermentable media, meiotic cell division, and cell wall biogenesis. (13, 14).Get3 is the ATPase subunit of the Get complex. It is also involved in resistance to heat and metal stress (arsenite transport) (15). Get3-GFP forms dot-like

structures in conditions of stress (15). Also, it has been shown that most of the Get complex shifts from the ER to dot-like structures under conditions of stress e.g. shift from rich to minimal medium (10). This suggests that the Get complex might have a role in stress response and its major functions have to be explored.

Objectives

Based on the above background, the following are the main objectives of this work:

1. Since *get2* mutation has been shown to affect [*PSI*$^+$] stability (S. Muller, J. Patterson and Y. Chernoff, unpublished data; and 23), the question arises whether the other components of the Get complex i.e. *GET1* and *GET3* affect [*PSI*$^+$] stability.
2. *Get2* mutation has been shown to affect the maintenance of [*PSI*$^+$] prion. We have addressed the question whether *get2* mutation has this effect on prions other than [*PSI*$^+$].
3. Since the molecular chaperone Hsp104 plays an important role in the propagation of yeast prions, it will be studied whether *get2* mutation operates by altering/modulating the levels and/or activity of Hsp104.
4. Since *get2Δ* causes a defect in prion maintenance, it will be investigated whether the [*PSI*$^+$] prion instability is reflected in the size of the polymers in the *get2Δ* strain.
5. Other possible mechanisms for the effect of *get2* on [*PSI*$^+$] will be investigated.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

Strains

All yeast strains used in this study are listed in Table 2.1.

Table 2.1 **List of *Saccharomyces cerevisiae* strains used in this study**

Name (synonym)	Genotype	Reference
1-1-74-D694 (OT55)	[*PSI*$^+$] *MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52*	2
7-74-D694 (OT56)	[*PSI*$^+$] *MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52*	8
74-D694 (OT60)	[*psi*$^-$ *PIN*$^+$] *MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52*	8
GT17	[*psi*$^-$ *pin*$^-$] *MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52*	32
GT982	[*PSI*$^+$] *MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get2::HIS3*	23
GT905	[*PSI*$^+$] *MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52*	23
GT1060	[*psi*$^-$ *pin*$^-$] *MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get2::HIS3*	This study
GT1072	[*PSI*$^+$] *MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get2::HIS3*	This study
GT1074	[*PSI*$^+$] *MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get3::HIS3*	This study
GT1105	[*PSI*$^+$] *MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get1:: KAN*^{R}	This study
GT1107	[*PSI*$^+$] *MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get3:: HIS3*	This study
GT234	[*psi*$^-$ *pin*$^-$] *MATα ade1-14 leu2-3, 112 his3-Δ200(or -11, 15) lys2 trp1-Δ ura3-52*	32
GT1078	[*psi*$^-$ *PIN*$^+$] *MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get2::HIS3*	This study
GT1080	[*psi*$^-$ *pin*$^-$] *MATa ade1-14 his3-Δ200 leu2-3,112 trp1-289 ura3-52, get2::HIS3*	This study
BY4741(OT352)	*get1 (YGL020C)::KAN*^{R}	24 (obtained from Open Biosystems)

The most commonly used strains in this work are isogenic derivatives of yeast strain 74-D694, which differ only in their prion status. Strain 74-D694 ([*psi*$^-$*PIN*$^+$]) is also known as OT60. The weak [*PSI*$^+$*PIN*$^+$] strain OT55 and the strong [*PSI*$^+$*PIN*$^+$] strain OT56 were independently induced by overexpression of *SUP35* in strain OT60 (2). These strains can be distinguished by their respective phenotypes. Nonsense suppression of the *ade1-14*$_{\text{UGA}}$ mutation is more efficient in strain OT56, resulting in growth on –Ade medium after 3-4 days and a white/light pink color on YPD, compared to the 7-8 day incubation time for OT55 cells on –Ade, and a pink color on YPD. Strain GT17 is [*psi*$^-$*pin*$^-$] and was acquired by GuHCl treatment of strain OT56 as described previously (4). A second set of isogenic strains are derivatives of the strong [*PSI*$^+$*PIN*$^+$] diploid parent GT81 (16). GT234 (17) is a meiotic segregant of GT81, cured of [*PSI*$^+$] and [*PIN*$^+$] by GuHCl. GT982 was obtained by deleting *GET2* gene in the OT56 background (23). GT905 was obtained by the EMS Mutagenesis of OT56 and is resistant to [*PSI*$^+$] curing by Hsp104 overexpression (23). It has a point mutation in the *GET2* gene at codon 473 and is hereafter referred to as *get2-473*. GT1060 is a [*psi*$^-$*pin*$^-$] derivative of GT982. [*PSI*$^+$] was lost spontaneously as a result of a transformation. It was tested for [*PIN*$^+$] and found to be [*pin*$^-$]. GT1072 was obtained by deleting the *GET2* gene in the OT55 strain using the template plasmid pFA6a-His3MX6 (Table 2.2). GT1074 was obtained by deleting the *GET3* gene in the OT55 strain using the template plasmid pFA6a-His3MX6. GT1105 was obtained by deleting the *GET1* gene in the OT56 strain. This deletion was made by transforming OT56 with the PCR product obtained by amplifying the genomic DNA of the yeast strain BY4741 containing deletion in the *GET1* gene (ORF YGL020C) (24). GT1107 was obtained by deleting the *GET3* gene in the OT56 strain using the template plasmid pFA6a-His3MX6. GT1078 is a [*psi*$^-$*PIN*$^+$] derivative of GT982. [*PSI*$^+$] was lost spontaneously through incubation on complete synthetic medium and [*PIN*$^+$] status was determined through [*PSI*$^+$] induction test. GT1080 is a [*psi*$^-$*pin*$^-$] derivative of GT982. [*PSI*$^+$] was lost spontaneously through incubation on complete synthetic medium and [*PIN*$^+$] status was

determined through [PSI^{+}] induction test. Strain BY4741 containing a deletion in the *GET1* gene (ORF YGL020C) was obtained from the *Saccharomyces* Genome Deletion Consortium (24).

Plasmids

All plasmids used in this study are listed in Table 2.2.

Table 2.2 **List and description of plasmids used in this study**

Plasmid	Markers (*E. coli*/ *S. cerevisiae*)	Description	Plasmid type	Reference (s)
YEp13	AMP^R/ *LEU2*	Basic cloning vector	YEp	17
pSTR7	AMP^R/ *LEU2*	P_{SUP35}-*SUP35*	YEp	17
pLH105	AMP^R/ *LEU2*	P_{GPD}-*HSP104*	YCp	17
pFA6a-His3MX6	AMP^R/ *HIS3*	Template plasmid used for making gene deletion constructs.	YEp	22
pMCUP1	AMP^R/ *URA3*	Base vector for copper induction.	YCp	Lindquist lab
pU-CUP-SUP35	AMP^R/ *URA3*	Full length *SUP35* gene under P_{CUP}	YCp	Eugene
pL-Sp-SUPNM-GFP	AMP^R/ *LEU2*	P_{SUP35}- *SUP35NM* fused to GFP	YCp	26
pMCUP-Hsp104	AMP^R/ *URA3*	*HSP104* under P_{CUP}	YCp	Kavita
pRS316-GAL	AMP^R/ *URA3*	P_{GAL} inserted in polylinker of pRS316	YCp	17
pGAL104-URA3	AMP^R/ *URA3*	*HSP104* fused to P_{GAL}	YCp	35
pYS104	AMP^R/ *URA3*	P_{HSP104}-*HSP104* inserted into pRS316	YCp	6
pRS316	AMP^R/ *URA3*	Basic cloning vector	YCp	33
pmCUPNMsGFP	AMP^R/ *URA3*	SUP35-NM-GFP under P_{CUP}	YCp	Lindquist lab

YEp- yeast episomal plasmid (2μ) (high copy number)
YCp- yeast centromeric plasmid (1-3 copies per cell)

The multicopy 2μ DNA-based *LEU2* plasmid pSTR7 bearing the *SUP35* gene under its own promoter, (and matching and empty vector control YEp13) have been described previously (17). pLH105 plasmid contains the *HSP104* gene under the constitutively

active *GPD* promoter (17). pFA6a-His3MX6 is the template plasmid used for making gene deletion constructs. His3MX6 is the *HIS5+* gene of *Schizosaccharomyces pombe*, it complements *his3* mutants in *S. cerevisiae* (22). pmCUP1 is a base vector for copper induction. pU-CUP-SUP35 contains the full length *SUP35* gene under the copper-inducible promoter. pL-Sp-SUPNM-GFP contains the NM region of *SUP35* fused to GFP under the endogenous *SUP35* promoter (26). pmCUP-Hsp104 contains the *HSP104* gene under the copper-inducible promoter. pRS316-GAL (17) contains the galactose-inducible promoter in the basic cloning vector pRS316 (33). pGAL104-URA3 contains the *HSP104* gene under the galactose-inducible promoter (35). pYS104 contains the *HSP104* gene under the endogenous *HSP104* promoter (6).

Antibodies

Antibody to Hsp104 was obtained from Dr. Susan Lindquist and used in 1:5000 dilution. Secondary antibody (anti-rabbit) was obtained from Sigma-Aldridge and used in 1:5000 dilution. Antibody to Sup35C was obtained from Dr. David Bedwell and used in 1:2000 dilution. Secondary antibody (anti-rabbit) was obtained from Sigma-Aldridge and used in 1:6000 dilution.

METHODS

Molecular biology techniques

Standard protocols were used for DNA electrophoresis and bacterial transformation (18).

E. coli plasmid DNA isolation

For large scale/maxi prep isolation of plasmid DNA, standard laboratory protocols were used (18). Fresh bacterial colonies were suspended in 250 ml of Luria

broth (LB) (10g/l tryptone, 5g/l yeast extract, 10g/l NaCl, pH 7.0) contained in a 1 liter flask, plus an appropriate antibiotic (i.e. Ampicillin) for plasmid selection. Cells were grown to an optical density (OD) reading of 0.8 at 550 nm (OD_{550}). Cells were transferred to sterile plastic bottles, and pellets were collected at 7000 rpm. Pellets were suspended in 10 ml Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0), were transferred to sterile Oak Ridge tubes, and were respun at 7000 rpm. Pellets were resuspended in 4.5 ml of Solution I plus lysozyme (0.5 ml of 20mg/ml lysozyme), incubated at room temperature for 10 minutes, then were placed on ice for 20 minutes, after which 10 ml of freshly prepared Solution II (0.2N NaOH, 1% SDS) was added, and cells were returned to ice for an additional 15 minutes. 7.5 ml of sodium acetate (3 M, pH 5.0) was added and cells were incubated on ice for 1 hour. Cell debris was pelleted at 14,000 rpm at 4°C for 20 minutes. Supernatants were collected into sterile tubes, and 20 ml isopropanol was added and samples were incubated for 20 minutes at room temperature, followed by 12,000 rpm spin for 15 minutes, and a wash with 70% ethanol. Dried pellets were resuspended in 4.0 ml TE (pH 8.0) and after DNA was completely dissolved, 4 ml LiCl (9 M) was added and the samples were transferred to -20°C for at least 20 minutes. The suspension was separated by centrifugation at 12,000 rpm for 20 minutes, and supernatants were transferred to sterile tubes to which 8 ml of 95% ethanol was added. Tubes were placed on ice for 1 hour, followed by collection of pellets at 12,000 rpm for 15 minutes. DNA pellets were washed with 70% ethanol, were dried, and resuspended in TE (pH 7.4).

Yeast and *E. coli* transformation procedures

All yeast transformations were performed according to lithium-treatment procedure described previously (19, 20). All *E. coli* transformations were performed using chemically competent *E. coli* cells according to standard laboratory protocols (18).

Standard yeast media and growth conditions

Yeast cultures were grown at 30°C unless otherwise noted. Standard yeast media and standard procedures for yeast cultivation and transformation were used (20). Cells were counted using a hemacytometer (Brightline). Synthetic media lacking adenine, histidine, leucine, or uracil are designated as –Ade, -His, -Leu, and -Ura, respectively. In all cases when the carbon source is not specifically indicated, 2% glucose (Glu) was used. 2% galactose (Gal) or 2% galactose and 2% raffinose (Gal+Raf) instead of glucose was used to induce *GAL* promoter. Liquid cultures were grown with at least a 1/5 liquid/flask volume ratio in a shaking incubator (200-250 rpm).

Yeast DNA isolation

Plasmid and genomic DNA from yeast cultures was collected according to standard laboratory protocols (20). Briefly, cells from late log phase cultures were centrifuged at 2000 rpm, and cell pellets were resuspended in 500 ul of 1M sorbitol, 0.1 M EDTA, pH 7.5 containing 4% of a 4 mg/ml lyticase solution and were incubated at 37° C overnight. Cells were briefly spun down at 14,000 rpm, and pellets were resuspended in 500 ul of a 50 mM Tris-HCl (pH 7.4), 20 mM EDTA solution. SDS was added to a final concentration of 10%, and the samples were incubated at 65°C for 30 minutes. 0.2 ml of 5 M potassium acetate was added and samples were placed on ice for 1 hour. Following 14,000 rpm centrifugation, 0.75 ml isopropanol was added to the supernatants, samples were centrifuged at 14,000 rpm for 5 minutes. Supernatants were discarded and pellets were washed with 70% ethanol, dried, resuspended in 0.4 ml TE (pH 7.4) plus 22 µl of a 1 mg/ml solution of RNAse A, and incubated at 37°C for 30 minutes. An equal volume of chloroform: isoamylalcohol (24:1) was added, and after centrifugation at 14,000 rpm for 1 min, $1/10^{th}$ volume of 3M sodium acetate (pH 5.0) was added to the top aqueous layer. DNA was precipitated with 2 volumes of 95% ethanol. Samples were

centrifuged at 14,000 rpm for 15 minutes, and pellets were washed with 70% ethanol. DNA pellets were dried and resuspended in 50 µl TE (pH 7.4) or water (if used for PCR).

Yeast protein isolation and analysis

Collection and analysis of yeast total protein lysates was conducted using standard yeast laboratory procedures (20, 18). Analysis of yeast total lysates was done according to previously published procedures (21), with slight modification. Yeast cells suspended in lysis buffer (100 mM Tris-HCl, pH7.5 + 200 mM NaCl + 1 mM EDTA + 5% glycerol + 0.5 mM DTT + 20 mM PMSF) were lysed with glass beads on a vortex mixer. Cell debris was removed by centrifugation at 3,000 rpm to produce a "total lysate" fraction. Resulting samples were heated at 95^{o}C for 10 min and run on the standard SDS-polyacrylamide gel. For performing the protein assays, gels were transferred onto Hybond ECL nitrocellulose membranes and reacted to the appropriate antibodies. Densitometry measurements were obtained from exposed films using the program Alphaimager 2000, Alpha Innotech Corporation.

Nonsense suppression assay for presence of [*PSI*$^{+}$]

Presence of [*PSI*$^{+}$] was detected by its ability to suppress the *ade1-14*$_{\text{UGA}}$ mutant allele, as described previously (8). The [*psi*$^{-}$] *ade1-14*$_{\text{UGA}}$ strains are not able to grow on medium lacking adenine (-Ade) and exhibit dark red color on rich (YPD) medium, while [*PSI*$^{+}$] *ade1-14*$_{\text{UGA}}$ strains are able to grow on –Ade and exhibit white or light-pink color on YPD.

Assay to monitor for presence of [*PIN*$^{+}$]

The [*PIN*$^{+}$] prion induces *de novo* formation of [*PSI*$^{+}$] in the presence of Sup35 overproduction (4). [*PIN*$^{+}$] is the prion form of the Rnq1 protein. The presence of [*PIN*$^{+}$] was monitored by the following assay: [*psi*$^{-}$] strains were mated to the strain [*psi*$^{-}$ *pin*$^{-}$]

GT234 bearing the multicopy *SUP35* plasmid pSTR7 (or YEp13 empty vector control). The [PIN^+] diploids, in contrast to [pin^-] diploids, grew on medium lacking adenine after 10-14 days of incubation due to *de novo* induction of [PSI^+].

Thermotolerance assay

Thermotolerance assay was done as described previously (6). 20 ml cultures were grown in 125 ml flasks in SD+13 or YPD media at 25°C for two hours to reach an OD of 0.8-1.0. The control set was directly kept on ice, the basal thermotolerance set was grown at 25°C for another 30 min, and the induced thermotolerance set was incubated at 37°C for 30 min. 500 µl aliquots each of both the thermotolerance sets were then incubated in 50°C water bath for 5, 10, 15 and 20 min and immediately moved to ice. 4 µl of cells from all the sets were then spotted on YPD plates in serial dilutions as 10^0 (undiluted), 10^{-1} (diluted 10 times), 10^{-2} and 10^{-3}. Image was taken after 2 days of growth at 30°C.

SDD-AGE

Semi-Denaturing Detergent Agarose Gel Electrophoresis (SDD-AGE) for the analysis of prion polymer sizes was performed as described previously (25) with slight modifications. 2-3 ml precultures were grown overnight in Oakridge tubes and diluted to 10^6 cells/ml cultures in flasks maintaining a 1:5 ratio between culture volume and the flask volume. The cultures were grown at 30°C with shaking at 200rpm or higher till the OD reached the exponential phase specific to the strain (refer to growth curves in Fig. 5.1). The cells were collected at 3000 rpm for 10 min and washed in 300 µl of complete buffer (1 Roche complete tablet in 2ml of sterile H_2O). The cells were frozen at -80°C for 20 min to break the cell wall and then resuspended in an appropriate volume of lysis buffer (25 mM Tris- HCI pH 7.5, 100 mM NaCl, 1 mM DTT, 10mM EDTA, 2 mM PMSF and proteinase inhibitors) depending on the size of the pellet. After adding 1/3 volume of acid-washed glass beads, the cells were vortexed 9 times at high speed for 20

sec each time (total of 3 min) with 60 sec on ice between vortexing. This was followed by centrifugation at 3000 rpm for 2 min. The supernatant was taken and protein concentration was measured using Bradford assay. ~120µg of protein was mixed with 1/3 vol. of 4X sample buffer (0.5x TAE (40 mM Tris-acetate, 1 mM EDTA), 2% SDS, 5% Glycerol and Bromophenol blue) and incubated at 37°C for 10 min. After incubation, the protein sample was loaded on the SDD-AGE gel (horizontal 1.8% agarose gels in 1X Tris-acetate-EDTA buffer with 0.1% SDS). Electrophoresis was performed using the running buffer (1X Tris-acetate-EDTA, 0.1% SDS) at 70-80 volts for 1 hour. Then, the gel was run at 90 volts until the dye reached the bottom. Western blotting was done using PVDF membrane using the tank electroblotting system (Biorad). Before transfer, the PVDF was incubated for 2-5 min in methanol and then in transfer buffer (15% methanol, 0.08% SDS, 5.8 g/L Tris, and 2.8 g/L Glycine) two times for 5 min each. Also, the gel was incubated in transfer buffer for 2 X 15 min on the shaker before the transfer. The electrophoresis and transfer were performed at 4°C.

Pringle method for gene deletion and tagging

For making gene deletions with a *HIS* marker, plasmid pFA6a-His3MX6 was used as described previously (22). His3MX6 is the *HIS5+* gene of *Schizosaccharomyces pombe*, it complements *his3* mutants in *Saccharomyces cerevisiae*. A construct for making gene deletion was prepared by using a template DNA. PCR was performed using this template and a pair of primers which contain regions which are homologous to the flanking regions of the gene of interest (Fig. 3.1). After transformation, gene deletion takes place due to homologous recombination with this construct. As shown in Fig. 3.2, gene deletion was identified on 0.8% agarose gel after doing PCR using another set of primers. The sequences of primers for making and checking *GET* gene deletions are shown in Table 2.3:

Table 2.3 **List and description of primers used in this study**

GET1	
Deletion*	F: 5'AGTACTCGTGACCAAATCTG3'
	R: 5'GGCTAGATCATCCACTCTTT3'
Checking	F: 5'GCAATCCTTGAACTACGTCT3'
	R: 5'AAAAGATTGGAGACGGAGGA3'
GET2	
Deletion	F:5'CTCTTCCATGTTTGTAGCATCAGCAACGTAGCTCTAGGAACGGATCCCCGGGTTAATTAA3'
	R:5'CCTGAAAAGAAAGCCGGGAATAATGTCGGGTTATGAGAACGAATTCGAGCTCGTTTAAAC3'
Checking	F: 5'CGTTTGCTGGGACAAAAGAA3'
	R: 5'GATTTTTCACGAACTCATCGC3'
GET3	
Deletion	F:5'AAACGTACGACAAGAACAAGAAGATCATCACATTGTAATTCGGATCCCCGGGTTAATTAA3'
	R:5'TTATATGTCGTATGTATCTATTTATGGTATTCAGGGGCTTGAATTCGAGCTCGTTTAAAC3'
Checking	F: 5'AAACGTACGACAAGAACAAG3'
	R: 5'CACACACATACCATCGTATT3'
*GET2***	
Tagging	F:5'TTCGTTTGTGCTAATTGTCTTAGGCTTATTGACGTACTTACGGATCCCCGGGTTAATTAA3'
	R:5'TTATGAGAACAATGTATTATATTACTGAACTATCTAGAATGAATTCGAGCTCGTTTAAAC3'
Checking	F: 5'GAGGCGGAAAAGCGCAGGTT3'
	R: 5'GCCGGGAATAATGTCGGGTT3'

*: The template DNA for making the deletion construct was the genomic DNA of strain BY4741 which contains a deletion in the *GET1* gene.

**: For making tagged *GET2* constructs, plasmids pFA6a-GFP(S65T)-HIS3MX6 and pFA6a-3HA-His3MX6 were used for placing GFP and HA tags respectively in frame at the 3' end of the *GET2* gene as described previously (22).

Fluorescence microscopy

Cells from liquid cultures or plates were taken directly and applied to slides for immediate visualization. Cells on slides were sealed with a coverslip using clear nail polish. The samples were scanned using an Olympus BX41 fluorescence microscope and image analysis was conducted using the Olympus DP71 camera.

The behavior of Sup35-GFP fusion proteins is closely linked to the existing prion state of the cell. 100 μm $CuSO_4$ was used to induce expression of *CUP1*-regulated constructs. Whenever the frequency of cells containing GFP aggregates was tabulated, at least 100 cells were counted in each case.

CHAPTER 3

EFFECT OF THE OTHER COMPONENTS OF THE GET COMPLEX (*GET1* AND *GET3*) ON [*PSI*$^+$] STABILITY

Since *get2* was shown to have an effect on [*PSI*$^+$] stability (S. Muller, J. Patterson and Y. Chernoff, unpublished data), deletions were made in the other components of the Get complex i.e. *GET1* and *GET3* to investigate whether they too influence the stability of [*PSI*$^+$] prion. Deletions were made in the OT56 background using the Pringle method (22) as explained in Chapter 2. The sequences of primers are given in Table 2.3. A schematic description is given in Fig. 3.1.

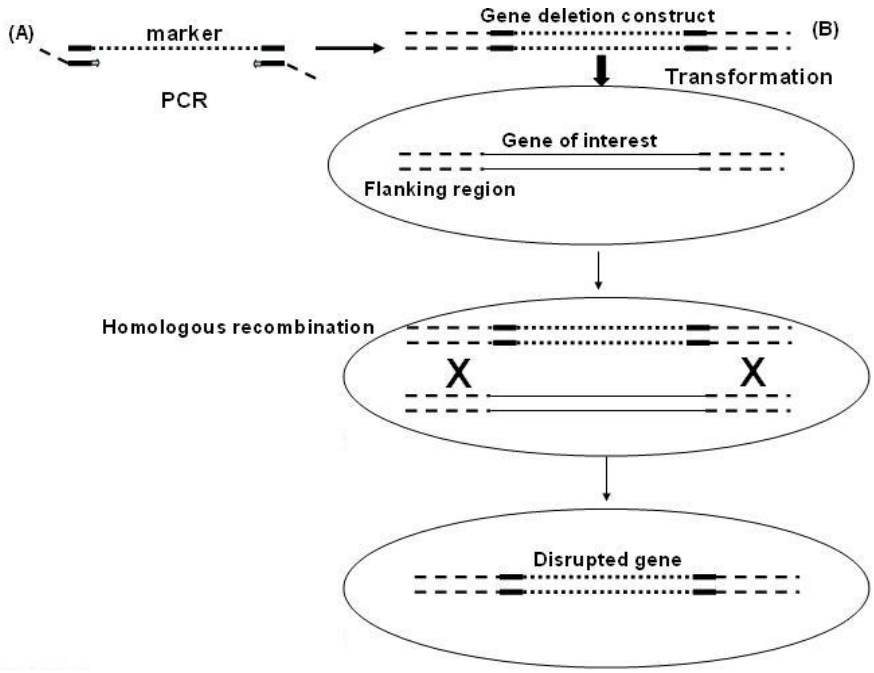

Figure 3.1 **Pringle method for making *GET* gene deletions**
The procedure for making gene deletions is carried out in two steps. **A.** A construct for making gene deletion is prepared by using a template plasmid pFA6a-His3MX6 (Table 2.2) or genomic DNA from BY4741 (Table 2.1). PCR is performed using this template and a pair of primers which are homologous to the plasmid or genomic DNA (both of which contain a marker) and contain regions which are homologous to the flanking regions of the gene of interest. These primers are listed in Table 2.3 as deletion primers for different genes. **B.** This PCR product is transformed into the wild type strain. Homologous recombination takes place with the flanking regions of the gene of interest as a result replacing it with the marker. The disruptants are selected on an appropriate selective medium. The presence of gene deletion can be confirmed by doing PCR with primers listed in Table 2.3 as checking primers for different genes.

A construct for making gene deletion is prepared by using a template DNA. PCR is performed using this template and a pair of primers which contain regions which are homologous to the flanking regions of the gene of interest. After transformation, gene deletion takes place due to homologous recombination with this construct. As shown in Fig. 3.2, gene deletion can be identified on 0.8% agarose gel after doing PCR using another set of primers (chapter 2). Using primers to detect *get1* gene deletion (Fig. 3.2A), wild type shows a 0.9 kb band whereas a deletion shows 1.88 kb band. As a control, genomic DNA from the collection strain used for creating the gene deletion construct (chapter 2) was also checked by PCR. It is an aneuploid and shows both wild type and deletion bands. For detecting *get3* gene deletion using plasmid pFA6a-His3MX6 (Fig. 3.2B) another set of primers were used showing wild type (1.18 kb) and deletion (1.43 kb) bands of different sizes.

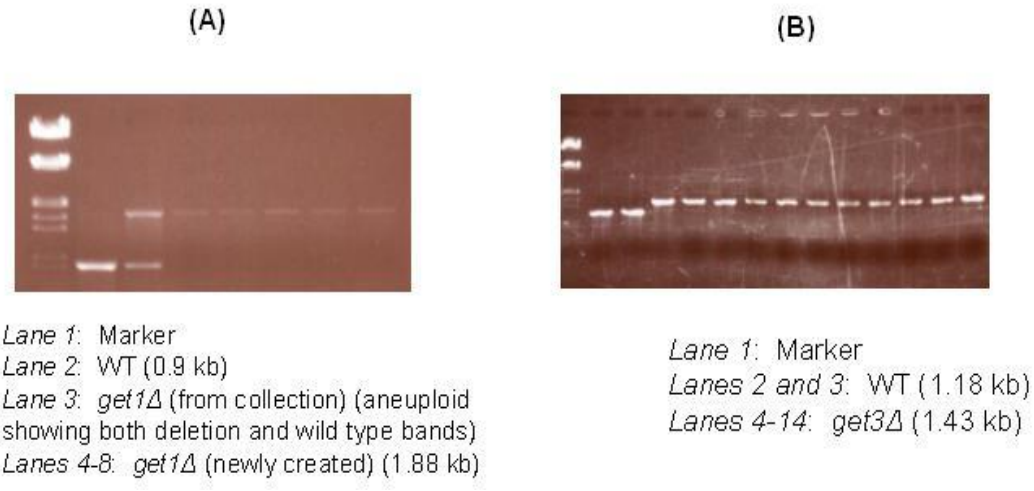

Figure 3.2 **The confirmation of *get1* and *get3* deletion constructions in strong [*PSI*$^{+}$] background by PCR**

The strong [*PSI*$^{+}$] strain (OT56) was transformed with PCR products having homologous regions to the genes of interest. These products were prepared by amplifying *GET1* gene from the genome of the *get1Δ* strain found in the collection of yeast gene deletion strains (see materials and methods and fig. 3.1) in (A), and pFA6a-His3MX6 plasmid (see materials and methods and Fig. 3.1) in (B). Disruption of genes was checked using another set of primers and confirmed by the fragment sizes on 0.8% agarose gel.

Curing defect in the presence of excess Hsp104

First, it was studied whether there was any curing defect in these strains in the case of Hsp104 over expression. The *get1Δ* and *get3Δ* strains along with the wild type control i.e. OT56 were transformed with plasmid pLH105 (Table 2.2). This plasmid contains *HSP104* gene under the constitutively active P_{GPD} promoter. The transformants were selected and patched on –Leu media. These plates were then replica-plaed to –Ade and –Leu-Ade media to detect the curing of [PSI^+] prion by lack of growth on medium lacking adenine (as explained in chapter 2).

As seen in Fig. 3.3, in the case of both *get3* and *get1* gene deletions [PSI^+] loss is decreased in the presence of excess Hsp104. This effect is similar to the effect previously observed for *get2* deletion. This experiment was also done with another plasmid where *HSP104* is under the Galactose inducible promoter (pGAL104-URA3) and similar results were obtained (not shown here).

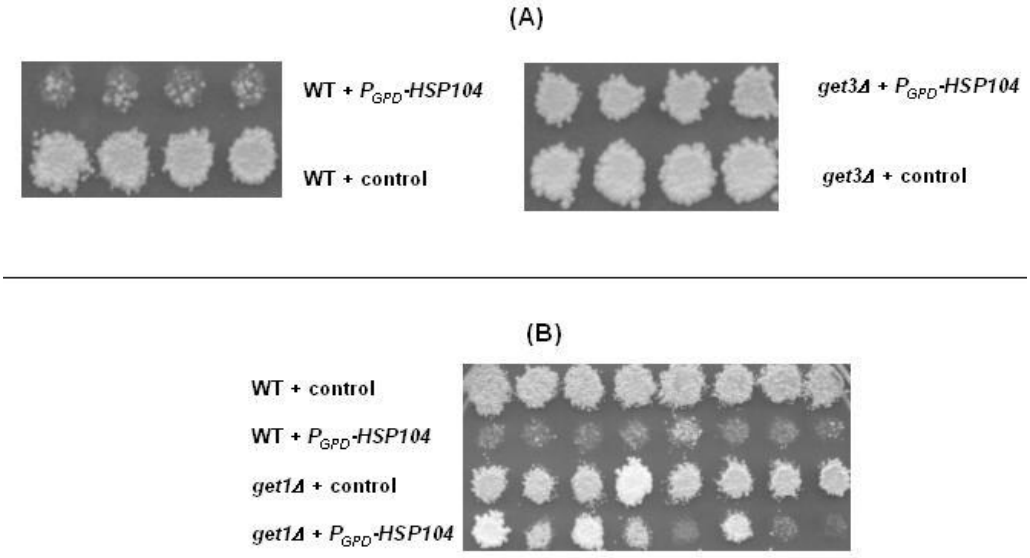

Figure 3.3 **[*PSI*$^+$] curing defect by excess Hsp104 is observed in *get3Δ* and *get1Δ***
A. Both wild type and *get3Δ* strains were transformed with pLH105 (P_{GPD}-*HSP104)* or control plasmids. The transformants were patched on –Leu and replica plated to –Leu-Ade media. Image was taken after ~10 days of incubation. Wild type strain is cured of the [*PSI*$^+$] prion by Hsp104 overexpression as seen by the lack of growth on media lacking Adenine. On the other hand, the isogenic *get3Δ* strain is not cured by excess Hsp104 and shows a [*PSI*$^+$] curing defect. **B.** The experiment described in A was repeated for wild type and *get1Δ* strains. Curing is observed in wild type whereas in some of the transformants of *get1Δ*, a curing defect is observed.

Spontaneous loss of [*PSI*$^+$] on synthetic media in the *get1* and *get3* deletion strains

Experiments were also performed to analyze [*PSI*$^+$] maintenance in the *get1Δ* and *get3Δ* strains through incubation on complete synthetic medium. The strains were streaked out on complete synthetic medium and several colonies were patched on another synthetic medium plate. This plate was then replica-plated to another synthetic medium plate and to YPD. Also, several colonies from the replica-plated synthetic medium plate were streaked out on YPD. In parallel, serial passages were also performed on YPD as a control. After such serial passages on synthetic media, the number of [*psi*$^-$] colonies was estimated based on red or dark pink color on YPD. Preliminary results suggest that some [*PSI*$^+$] instability is observed in the *get3Δ* strain as well, but not at the same level as seen in the *get2Δ*. However, *get1Δ* does not cause any [*PSI*$^+$] instability (Fig. 3.4 and table 3.1). More sensitive experiments are needed to analyze the effect of deletion of *GET1* and *GET3* genes on [*PSI*$^+$] stability.

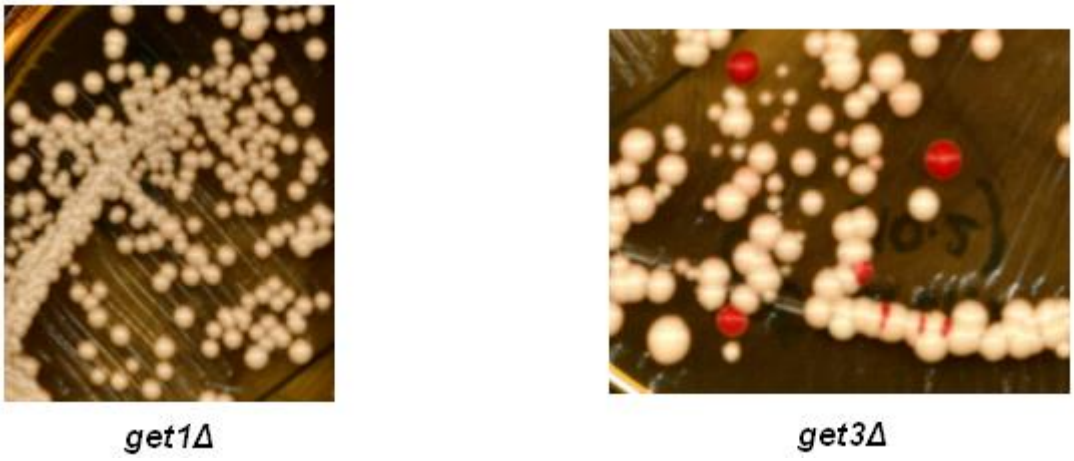

Figure 3.4 **Effect of *get1Δ* and *get3Δ* on [*PSI*$^+$] stability**
The strains were streaked out on YPD after growth on complete synthetic medium (~3-4 days). Image was taken after 2 days of incubation on YPD. [*PSI*$^+$] loss was studied by the appearance of red colonies on YPD which spontaneously lose [*PSI*$^+$]. *get1Δ* does not cause any [*PSI*$^+$] instability whereas *get3Δ* causes some instability on synthetic medium. See table 3.1.

Table 3.1 **Effect of *get1Δ* and *get3Δ* on [*PSI*$^+$] stability**

Strain	Ade$^+$	Ade$^-$	Total	**% Ade$^-$**
Wild type	100	0	100	0
get1Δ	100	0	100	0
get3Δ	100	14	114	12.3

See Fig. 3.4

Conclusion

Deletion of *get3* or *get1* exhibits a defect in [*PSI*$^+$] curing by excess Hsp104 similar to that in *get2* mutants. The above data suggests that the Get complex affects prion maintenance not only through Get2 but through a common function of the Get complex. There might be some effect of *get3* on [*PSI*$^+$] stability as well, as suggested by preliminary data. However, the instability observed is not as strong and more sensitive experiments are needed to study the same.

CHAPTER 4

EFFECT OF *GET2* ON PRIONS OTHER THAN [*PSI*$^+$]

Since *get2* affects [*PSI*$^+$] maintenance (S. Muller, J. Patterson and Y. Chernoff, unpublished data), it was interesting to study whether it has an effect on the stability of other prions as well.

Spontaneous loss of [*PIN*$^+$] in the *get2* deletion strain

Experiments were conducted to study the maintenance of [*PIN*$^+$] prion in *get2Δ* strain through incubation on complete synthetic medium as explained in Chapter 3. After incubation on synthetic medium, the number of [*psi*$^-$] colonies was estimated based on red or dark pink color on YPD. These [*psi*$^-$] colonies were saved on complete medium for [*PSI*$^+$] induction test as described in Chapter 2 and Fig. 4.1A. As shown in the Table 4.1 and Fig 4.1B, the proportion of [*pin*$^-$] colonies was more as compared to those that were [*PIN*$^+$].

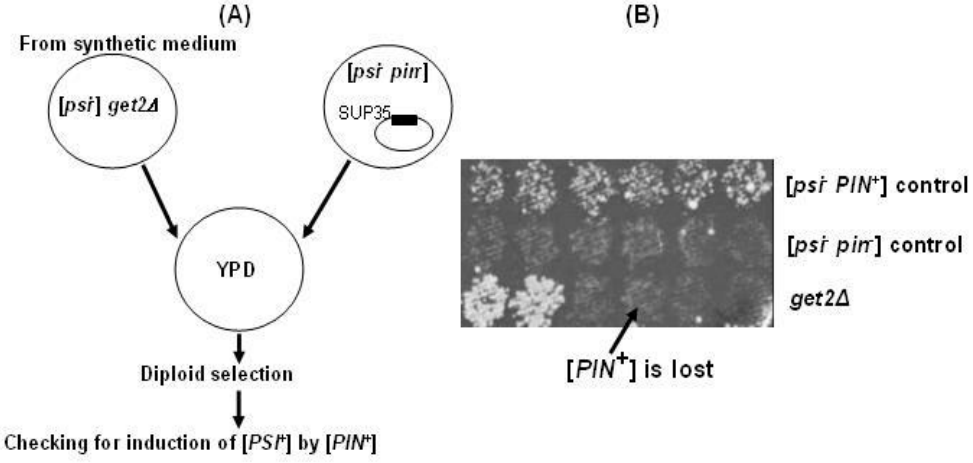

Figure 4.1 ***Get2* deletion strain exhibits spontaneous loss of [*PIN*$^+$] prion**
A. The scheme of the [*PSI*$^+$] induction test used to determine the presence of [*PIN*$^+$] prion in the [*psi*$^-$] *get2* deletion strain. This strain picked up from various time points of the serial passage experiment, is mated on rich media (YPD) with a [*psi*$^-$ *pin*$^-$] strain containing pSTR7 (multicopy *SUP35*) plasmid. After selecting for diploids on synthetic medium selective for diploids, the plate was replica plated to synthetic medium without adenine selective for diploids. Image was taken after ~10 days and the presence of [*PIN*$^+$] was detected through the *de novo* induction of [*PSI*$^+$] which leads to growth on media lacking adenine. **B.** *Get2* deletion strain was incubated on complete synthetic media for several passages. From each time point several [*psi*$^-$] colonies were picked up and used in [*PSI*$^+$] induction test to determine their [*PIN*$^+$] status. More [*psi*$^-$] colonies were [*pin*$^-$] as compared to [*PIN*$^+$]. This was determined by the lack of growth of the diploid on media lacking adenine indicating that no [*PSI*$^+$] induction took place. Image was taken after ~10 days.

Table 4.1 ***Get2* deletion strain exhibits spontaneous loss of [*PIN*$^+$] prion**

Round of incubation	**[*psi*$^-$] *get2Δ* colonies tested**		
	[*pin*$^-$]	**[*PIN*$^+$]**	**Total**
1	10	9	19
2	7	6	13
3	11	11	22
4	9	6	15

See figure 4.1 for experimental scheme.

De novo induction of [*PSI*$^+$]

Also, *de novo* induction of [*PSI*$^+$] in the [*psi*$^-$ *PIN*$^+$] *get2Δ* colonies was studied. [*psi*$^-$ *PIN*$^+$] wild type and [*psi*$^-$*PIN*$^+$] *get2Δ* were transformed with pU-CUP-*SUP35* (*SUP35* under copper promoter) or control plasmids. Transformants were selected and patched on –Ura and incubated for ~4 days. This was followed by replica plating to –Ura plates with or without 100 µM CuSO4. After incubation for ~4 days, *de novo* induction of [*PSI*$^+$] was analyzed by growth on medium lacking adenine. *De novo* [*PSI*$^+$] induction was not affected in *get2Δ* as compared to wild type (Fig. 4.2).

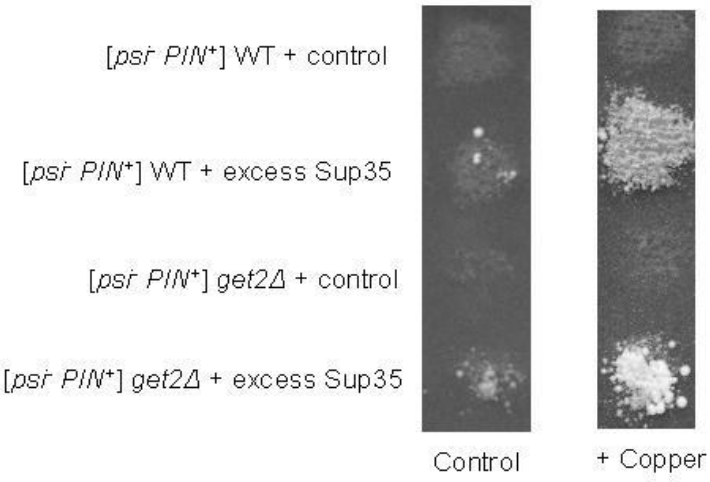

Figure 4.2 ***De novo* induction of [*PSI*$^+$] is not affected in *get2Δ***
[*psi*$^-$*PIN*$^+$] wild type and [*psi*$^-$*PIN*$^+$] *get2Δ* were transformed with pU-CUP-*SUP35* (*SUP35* under copper promoter) or control plasmids. Transformants were selected and patched on –Ura and incubated for ~4 days. This was followed by replica plating to –Ura plates with or without 100 µM $CuSO_4$. After incubation for ~4 days, these plates were then replica plated to synthetic medium selective for the plasmids and lacking adenine. Images were taken after ~10 days of incubation. De novo induction of [*PSI*$^+$] is seen by growth on medium lacking adenine. Induction is not affected in *get2Δ* as compared to wild type.

Effects of *get2* and *get3* deletions in a weak [*PSI*$^+$] background

Get2 and *get3* genes were also deleted in the weak [*PSI*$^+$] background i.e. OT55. OT55 was transformed with PCR products having homologous regions to the genes of interest. This product was prepared by using pFA6a-His3MX6 template plasmid and primers (see materials and methods and Fig. 3.1). Disruption of genes was checked using another set of primers and confirmed by the fragment sizes on 0.8% agarose gel. The fragment sizes for *get2Δ* were 1.1 kb (wild type) and 1.53 kb (deletion) whereas the fragment sizes for *get3Δ* were 1.18 kb (wild type) and 1.44 kb (deletion) (Fig. 4.3).

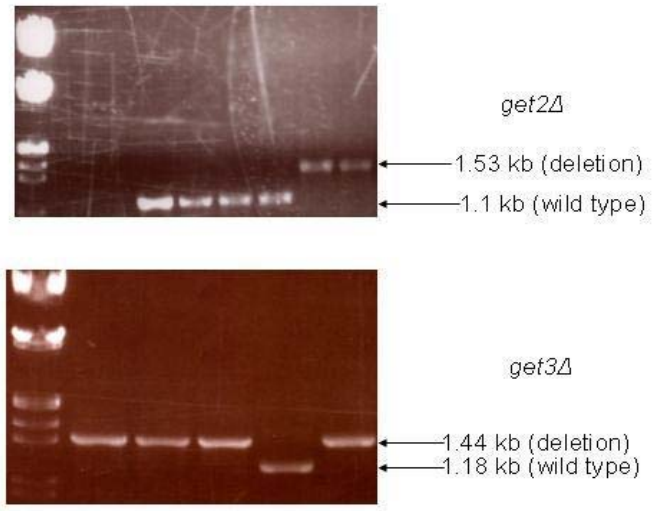

Figure 4.3 **The confirmation of *get2* and *get3* deletion constructions in weak [*PSI*$^+$] background by PCR**

The weak [*PSI*$^+$] strain (OT55) was transformed with PCR products having homologous regions to the genes of interest. This product was prepared by using pFA6a-His3MX6 plasmid and primers (see materials and methods and Fig. 3.1). Disruption of genes was checked using another set of primers and confirmed by the fragment sizes on 0.8% agarose gel.

These deletions were analyzed for the stability of [*PSI*$^+$] through incubation on complete synthetic medium as described in chapter 3. However, *get2* and *get3* deletions do not have any effect on [*PSI*$^+$] stability in a weak [*PSI*$^+$] background (Fig. 4.4 and table 4.2).

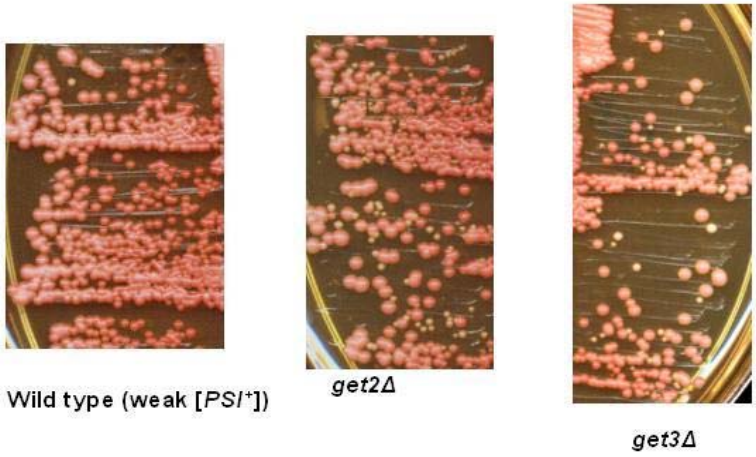

Figure 4.4 ***Get2* and *get3* deletions do not have an effect in weak [*PSI*$^+$] background**
The strains were streaked out on YPD after incubation on complete synthetic medium (~3-4 days). Images were taken after 2 days of incubation on YPD. [*PSI*$^+$] is stably maintained in both *get2* and *get3* deletions in a weak [*PSI*$^+$] background as seen by the absence of red colonies which depict loss of [*PSI*$^+$] prion. See table 4.2

Table 4.2 **Effects of *get2Δ* and *get3Δ* on [*PSI*$^+$] stability in weak [*PSI*$^+$] background**

Strain	Pink (Ade$^+$) colonies	Red (Ade$^-$) colonies	Total colonies	**% Ade$^-$**
Wild type (weak [*PSI*$^+$])	100	0	100	0
get2Δ	100	0	100	0
get3Δ	100	0	100	0
Strain	Ade$^+$	Ade$^-$	Total	**% Ade$^-$**
Wild type (strong [*PSI*$^+$])	100	0	100	0
get2Δ	50	46	96	47.9
get3Δ	100	14	114	12.3

See Fig. 4.4

As shown in table 4.2, *get2* and *get3* deletions do not have any effect on [*PSI*$^+$] stability in a weak [*PSI*$^+$] background. However, as discussed in chapters 1 and 3 respectively, *get2* and *get3* deletions affect [*PSI*$^+$] stability in a strong [*PSI*$^+$] background.

Conclusion

Get2 causes instability of both [*PSI*$^+$] and [*PIN*$^+$] prions on synthetic medium in a strong [*PSI*$^+$] background. *De novo* [*PSI*$^+$] induction is not affected in *get2Δ*. Also, *get2*

and *get3* deletions do not have any effect on [*PSI*$^{+}$] stability in a weak [*PSI*$^{+}$] background.

CHAPTER 5

DOES *GET2* OPERATE BY ALTERING/MODULATING HSP104 LEVELS AND/OR ACTIVITY?

Hsp104 levels

Since Hsp104 plays a role in prion propagation (Fig. 1.3), it was studied whether the effect of *get2* on prion maintenance is due to an alteration in the levels of the molecular chaperone Hsp104. Total protein extracts were prepared from wild type OT56, *get2Δ* and *get2-473* strains grown to the exponential and stationary phases of growth. Growth curves of these strains were obtained first by preparing 10^6cells/ml cultures in complete synthetic medium and recording the OD readings approximately every two hours. (Fig. 5.1).

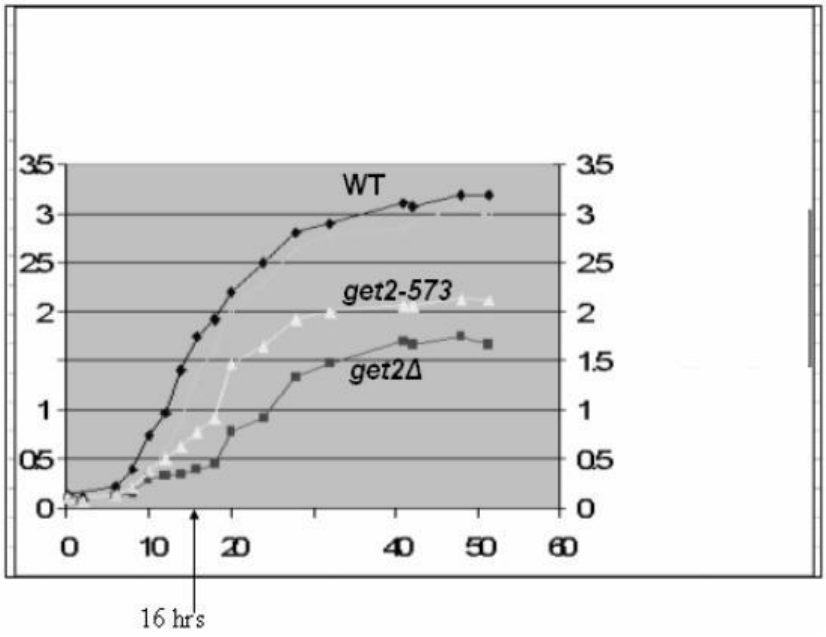

Figure 5.1 **Growth curves for wild type and *get2* mutants**
10^6 cells/ml cultures of wild type, *get2-473* and *get2Δ* strains were made in complete synthetic medium and growth curves were obtained by recording OD values approximately every two hours. At 16-hour time point, all three cultures were in exponential phase and protein extracts for Western blotting were prepared at this time point.

As seen in the figure, at the 16- hour time point all the strains were in the exponential phase of growth and reached the stationary phase at around 48 hours. After running the samples in polyacrylamide gel electrophoresis, Western Blotting was performed and the levels of Hsp104 were detected using antibody to Hsp104 as described in Materials and Methods. Fig. 5.2A shows the protein levels among the three strains. Endogenous Hsp104 levels show an increase in the *get2-473* and *get2Δ* strains as compared to those found in wild type. Ade 2 was used as a loading control.

After normalizing with the levels of Ade2 protein, it was found that the Hsp104 levels in the *get2* mutant strains were not decreased as compared to the wild type strain in the stationary phase either (not shown here).

Experiments were also done to compare the Hsp104 levels among the three strains in case of overexpression of this protein in the cell. All three strains were transformed with Hsp104 overexpression (pLH105) or control (YEp13) plasmids as described in table 2.2. Total protein extracts were prepared and the levels of Hsp104 were detected after Western blotting. Fig. 5.2B shows the Hsp104 levels in all the combinations. The fold induction of Hsp104 as compared to control for each strain are shown. *Get2Δ* strain shows significant increase in induction levels of Hsp104 as compared to wild type. *Get2-473* strain also shows an increase in Hsp104 induction levels.

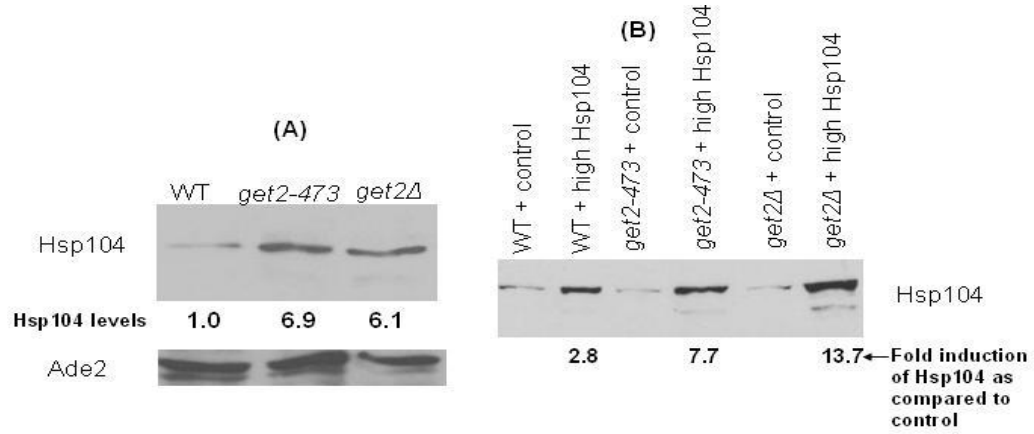

Figure 5.2 **The [*PSI*$^+$] curing and maintenance defects in the *get2* deletion and mutant strains are not due to a decrease in the Hsp104 levels**

A. Wild type, *get2-473* and *get2Δ* strains were grown in complete synthetic medium (SD+13) and total protein extracts were prepared from 10^6 cells/ml cultures grown to exponential phase (~16 hours) as shown in figure 5.1. The protein samples were run in polyacrylamide gel electrophoresis and Western blotting was done using Hsp104 antibody. Endogenous Hsp104 levels show an increase in the *get2-473* and *get2Δ* strains as compared to those found in wild type. Ade 2 was used as a loading control. The relative levels as shown by numbers below the blots were determined by densitometry. **B.** Wild type, *get2-473* and *get2Δ* strains were transformed with pLH105 (P_{GPD}-*HSP104)* or control plasmids and grown in –Leu media. Total protein extracts were prepared from cells grown to exponential phase and run in polyacrylamide gel electrophoresis. Western blotting was done using Hsp104 antibody. *Get2Δ* shows a significant increase in over expressed Hsp104 levels as compared to wild type.

Hsp104 activity analyzed by thermotolerance assay

Experiments were performed to analyze the activity of Hsp104 through Thermotolerance assay as described in Chapter 2. The mutants *get2-473* and *get2Δ* were analyzed along with the wild type strain in the Thermotolerance assay as described in Fig. 5.3. The strains were grown at 37°C for 30 min as a mild pre-heat treatment. After this they were given heat shock at 50°C for 5, 10, 15 and 20 min and spotted on complete medium plates to assess their viability.

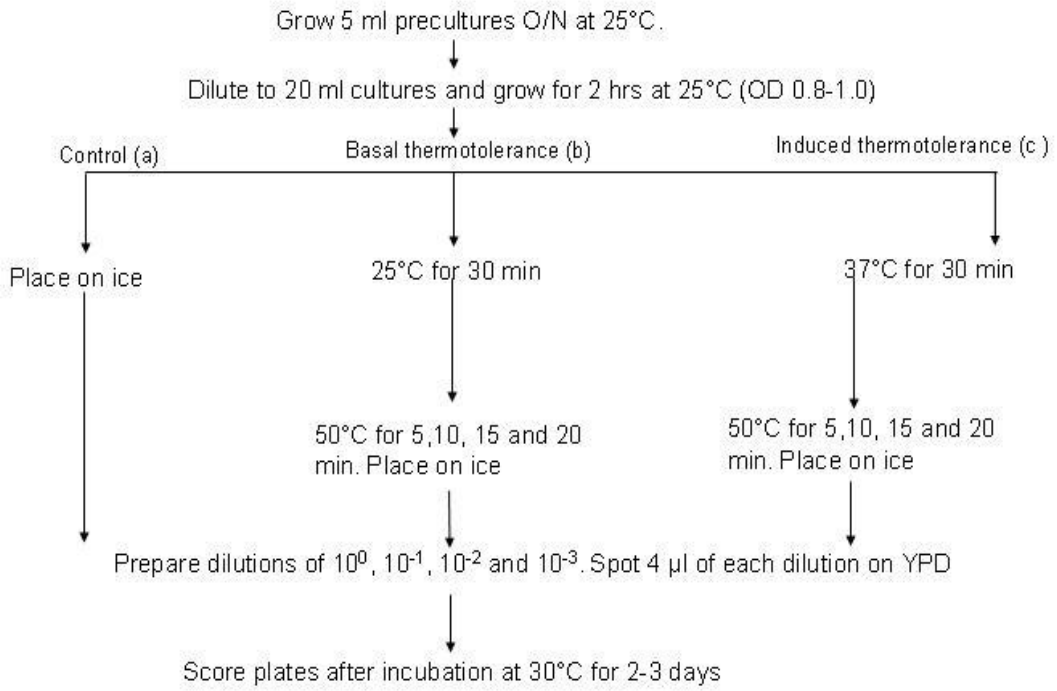

Figure 5.3 **The experimental scheme for thermotolerance assay**
Wild type, *get2-473* and *get2Δ* strains were grown in complete synthetic medium or YPD at 25°C for two hours to reach an OD of 0.8-1.0. The control set (a) was directly kept on ice, the basal thermotolerance set (b) was grown at 25°C for another 30 min, and the induced thermotolerance set (c) was incubated at 37°C for 30 min. Sets b and c were then incubated at 50°C for 10 min. 4 µl of cells from all the sets were then spotted on YPD in serial dilutions as 10^{0} (undiluted), 10^{-1} (diluted 10 times) and so on. Image was taken after 2 days of growth at 30°C.

Since the molecular chaperone Hsp104 is induced during the pre-heat treatment, it helps in the disaggregation of aggregated proteins during the heat shock treatment and thus increases cell survival. This is a measure of induced thermotolerance. As a measure of basal thermotolerance, no pre-heat treatment was given before the heat shock at 50°C. As seen in Fig. 5.4, in the complete synthetic medium, basal thermotolerance (partly Hsp104 dependent) is increased in the mutants as compared to wild type. Induced thermotolerance (strictly Hsp104 dependent) in the *get2-473* and *get2Δ* strains is comparable to the wild type. In the rich medium, induced thermotolerance in the *get2-473* and *get2Δ* stains is comparable to the wild type. Basal thermotolerance is comparable in all the three strains but is lower as compared to that in complete synthetic medium.

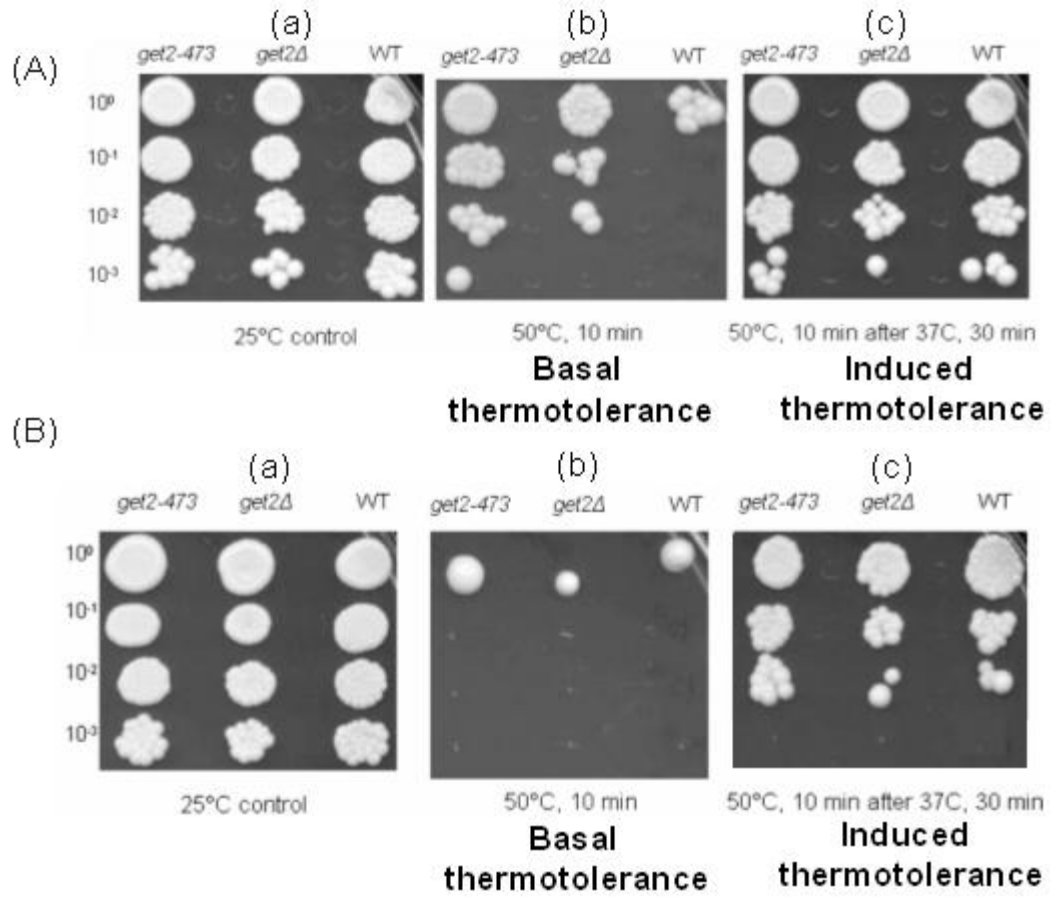

Figure 5.4 **The [*PSI*$^+$] curing and maintenance defects in the *get2* deletion and mutant strains are not due to a decrease in the activity of Hsp104**
A. Thermotolerance assay was done in complete synthetic medium as described in Fig. 5.3. Image was taken after 2 days of growth at 30°C. Basal thermotolerance (partly Hsp104 dependent) is increased in the mutants as compared to wild type (b). Induced thermotolerance (strictly Hsp104 dependent) in the *get2-473* and *get2Δ* strains is comparable to the wild type as seen in (c). **B.** Thermotolerance assay was done in YPD as described in Fig. 5.3. Image was taken after 2 days of growth at 30°C. As seen c, induced thermotolerance in the *get2-473* and *get2Δ* stains is comparable to the wild type. Basal thermotolerance is comparable in all the three strains (b) but is lower as compared to that in complete synthetic medium (A-b).

Conclusion

Since the Hsp104 levels are not decreased in the mutants as compared to wild type, the curing and maintenance defects in the *get2* mutants is not due to a decrease in Hsp104 levels. Also, as seen in the thermotolerance assay, the activity of Hsp104 is not decreased in the *get2* mutants as compared to the wild type. Thus the curing and maintenance defects are not due to a decrease in the activity of Hsp104 either. On the

other hand, basal thermotolerance in synthetic medium is increased in the mutants as compared to wild type. This corresponds to the results for the Hsp104 protein levels as seen by Western Blotting. The Hsp104 protein levels are higher in the mutants as compared to wild type.

CHAPTER 6

COMPARISON OF THE [*PSI*$^+$] AGGREGATE PATTERNS AND SIZES BETWEEN *GET2Δ* MUTANT AND WILD TYPE

Analysis of aggregate sizes using SDD-AGE

Since neither Hsp104 levels nor its activity are seen to be affected in the *get2Δ* strain, the next question to be asked was regarding the [*PSI*$^+$] aggregate size in the *get2Δ* strain. As the *get2Δ* strain shows some defects in prion maintenance, it was worth checking whether the [*PSI*$^+$] aggregates show any difference with respect to size as compared to that in wild type. Total protein extracts were prepared from the wild type and *get2Δ* strains and SDD-AGE was performed as described in materials and methods. Fig. 6.1A shows that the range of aggregate sizes is wider, and there are more of aggregates of larger sizes in *get2Δ* strain as compared to wild type. This experiment was repeated four times and similar results were obtained. Also, the result was unchanged when an additional high speed centrifugation (100,000g) was performed on the protein samples. The supernatant was then run on SDD-AGE. The huge aggregates of the *get2Δ* strain are not pulled down at high speed of centrifugation (Fig. 6.1B).

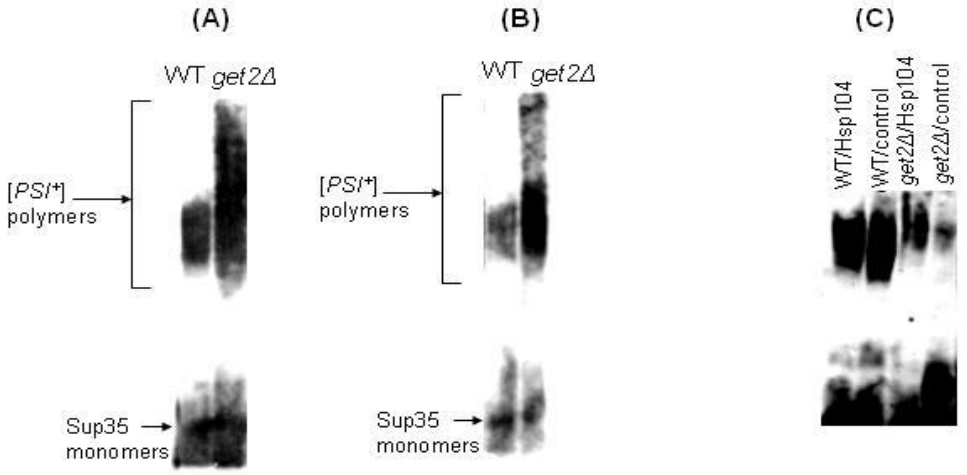

Figure 6.1 **The [*PSI*$^+$] polymer size is increased in the *get2Δ* strain as compared to the wild type A**. Wild type and *get2Δ* strains were grown in complete synthetic medium till the late exponential phase and total protein extracts were prepared as described in Materials and Methods. After normalizing by Bradford assay, ~120µg of protein with 2% SDS was loaded on SDD-AGE. After transferring the proteins to PVDF membrane, immunoblotting was performed using Sup35C antibody. The range of aggregate sizes is wider, and there is more of aggregates of larger sizes in *get2Δ* strain as compared to wild type. This experiment was repeated four times and similar results were obtained. **B.** The result was unchanged when an additional high speed centrifugation (100,000g) was performed on the lysates after adding 2% SDS to them. This increased the final concentration of SDS to 4% after adding the loading buffer, as compared to only 2% in A. The supernatant was then run on SDD-AGE. The huge aggregates of the *get2Δ* strain are not pulled down at high speed. **C.** Wild type, *get2Δ* and *get2-473* strains were transformed with plasmid containing *HSP104* gene under endogenous promoter or control plasmid. 10^6 cells/ml cultures were grown in –Ura media for ~24 hours and total protein extracts were prepared. SDD-AGE was performed using the protein lysates and immunoblotting was done using Sup35C antibody. The aggregates are bigger in wild type expressing excess Hsp104 as reported previously (Kryndushkin *et al*. 2003) and as discussed in chapter 6. The effect in *get2Δ* appears to be in the same direction. The variability in the experiment is discussed in chapter 6.

The aggregate sizes were also compared in the presence of excess Hsp104. Wild type, *get2Δ* and *get2-473* strains were transformed with *HSP104* under its endogenous promoter (pYS104) or control (pRS316) plasmids. The cultures were grown in selective medium and SDD-AGE was performed on total protein extracts (Fig. 6.1C). Wild type strain over expressing Hsp104 showed bigger aggregates as compared to wild type strain containing the control plasmid. This result agrees with that reported previously (25) that when Hsp104 is over expressed, the bigger aggregates resistant to Hsp104 action are selected for since they grow faster with an increasing pool of monomers. G*et2Δ* strain showed results in the same direction as wild type indicating that the difference in aggregate size is not due to ineffective action of Hsp104. In *get2-473* mutant, clear difference from wild type was not seen. Also, decreased range of polymer distribution in

get2Δ in Fig. 6.1C, compared to 6.1A and B, is probably due to less amount of protein loaded. However, the results were not completely reproducible due to the inherent variation in the experiment. This is possible due to the loss of plasmid from some cells during growth. These cells contribute to the total protein extract and cause variation in the experiment. Another reason could be the loss of [*PSI*$^{+}$] prion from some of the cells. More experiments are needed, possibly using galactose- inducible promoter for *HSP104* expression so that over expression of Hsp104 can be regulated for specific time-points to see the effect on aggregate sizes.

To study whether combining the wild type and mutant samples had any effect, another SDD-AGE experiment was performed (scheme described in Fig. 6.2A). 2% SDS was added to the protein extract from wild type which was then centrifuged at 100,000g and the supernatant was run on SDD-AGE. The protein extract from the [*psi*$^{-}$] *get2Δ* strain was centrifuged at 100,000g and the pellet was combined with the supernatant of the wild type strain. This combination was run on SDD-AGE and western blotting was done using the Sup35C antibody. As seen in Fig. 6.2B, the bigger aggregates of the *get2Δ* strain are not reconstituted in *trans*; the combination shows the same [*PSI*$^{+}$] aggregate size as wild type.

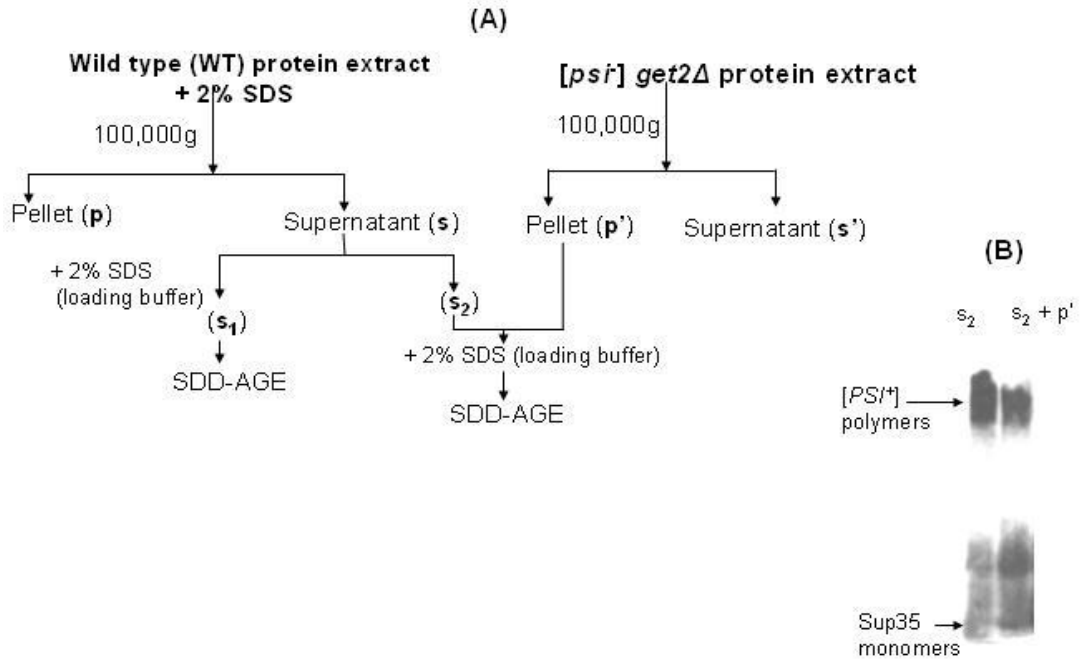

Figure 6.2 **The bigger aggregates of the *get2Δ* strain are not reconstituted in *trans***
A. 2% SDS was added to the protein extract from wild type which was then centrifuged at 100,000g and the supernatant was run on SDD-AGE. The protein extract from the [*psi*$^-$] *get2Δ* strain was centrifuged at 100,000g and the pellet was combined with the supernatant of the wild type strain. This combination was run on SDD-AGE and western blotting was done using the Sup35C antibody. **B.** The experiment was performed as described in A. The bigger aggregates of the *get2Δ* strain are not reconstituted in *trans*; the combination shows the same [*PSI*$^+$] aggregate size as the wild type.

Analysis of aggregates using fluorescence microscopy

Also, fluorescence microscopy experiment was performed to compare the pattern of aggregates between wild type and *get2-473* mutant. The strains were transformed with pmCUPNMsGFP plasmid (*SUP35NM-GFP* under copper inducible promoter) and high Hsp104 (pGAL104-URA3) or control plasmids. Transformants were selected on selective medium and replica plated to -Ura-His/Gal + $CuSO_4$ medium. Cells were taken directly from plates and observed under fluorescence microscope after 5 and 10 days of induction. In general, the percentage of cells containing visible aggregates was much lower in *get2-473* strain as compared to wild type (Tables 6.1 and 6.2). Also, more than 50% of *get2-473* aggregates in the presence of excess Hsp104 were rings/bars, which is significantly higher than that observed in wild type.

Table 6.1 Analysis of [*PSI*$^+$] aggregate pattern in solid medium in *get2-473* mutant as compared to wild type

Strain + Plasmid	No. of fluorescing cells	No. of cells with visible aggregates	% of cells with visible aggregates
WT+ CUP-Sup35-GFP + control	108	52	48.1
WT + CUP-Sup35-GFP + high Hsp104	130	48	36.9
get2-473+ CUP-Sup35-GFP + control	40	6	15
get2-473+ CUP-Sup35-GFP + high Hsp104	52	7	13.4

The data was recorded after 5 days of induction

Table 6.2 Analysis of [*PSI*$^+$] aggregate pattern in solid medium in *get2-473* mutant as compared to wild type

Strain/Plasmid	No. of fluorescing cells	No. of cells with visible aggregates	% of cells with visible aggregates
WT + CUP-Sup35-GFP + control	139	31	22.3
WT + CUP-Sup35-GFP + high Hsp104	100	20	20
get2-473+ CUP-Sup35-GFP + control	116	12	10.3
get2-473+ CUP-Sup35-GFP + high Hsp104	106	37	34.9

The data was recorded after 10 days of induction

This experiment was also performed in liquid medium repeating the transformations as above. 10^6cells/ml cultures were prepared in –Ura-His/Gal-Raf + 100μM $CuSO_4$ and cells were observed under the fluorescence microscope after 24 and 48 hours of induction. In general, the percentage of cells containing visible aggregates was much lower in *get2-473* strain as compared to wild type (Tables 6.3 and 6.4). Rings/bars were not observed in liquid medium.

Table 6.3 Analysis of [*PSI*$^+$] aggregate pattern in liquid medium in *get2-473* mutant as compared to wild type

Strain/Plasmid	No. of fluorescing cells	No. of cells with visible aggregates	% of cells with visible aggregates
WT + CUP-Sup35-GFP + high Hsp104	113	17	15
WT + CUP-Sup35-GFP + control	101	11	10.8
get2-473+ CUP-Sup35-GFP + high Hsp104	102	7	6.8
get2-473+ CUP-Sup35-GFP + control	100	8	8

Two transformants were analyzed for each combination after 24 hours of induction.

Table 6.4 Analysis of [*PSI*$^+$] aggregate pattern in liquid medium in *get2-473* mutant as compared to wild type

Strain/Plasmid	No. of fluorescing cells	No. of cells with visible aggregates	% of cells with visible aggregates
WT + CUP- Sup35-GFP + high Hsp104	106	75	70.7
WT + CUP- Sup35-GFP + control	102	21	20.5
get2-473+ CUP- Sup35-GFP + high Hsp104	105	29	27.6
get2-473+ CUP- Sup35-GFP + control	103	17	16.5

Two transformants were analyzed for each combination after 48 hours of induction.

Another fluorescence microscopy experiment was performed on cells expressing *SUP35-GFP* under its endogenous promoter. However, no systematic difference was observed between the mutant and wild type (data not shown).

Conclusion

The range of aggregate sizes is wider, and there are more of aggregates of larger sizes in *get2Δ* strain as compared to wild type. Also, these large aggregates are not pulled down at the high centrifugation speed of 100,000g.

CHAPTER 7

MODULATION OF THE EFFECTS OF *GET2* DEFICIENCY ON PRION BY CALCIUM CONCENTRATIONS

Effect of calcium on [*PSI*$^+$] instability

To explore other mechanisms for the effect of *get2* on prion propagation, we studied the effect of calcium on prion maintenance in *get2Δ* strain. Incubation of the *get2Δ* strain was done on three types of complete synthetic media. These were control, medium containing 10mM $CaCl_2$, and medium containing 780μM EGTA. The master plate was replica plated to these three different media so that the same patches can be compared from all the media. At each time point, several colonies were streaked out on YPD to assess [*PSI*$^+$] instability by color (Fig. 7.1).

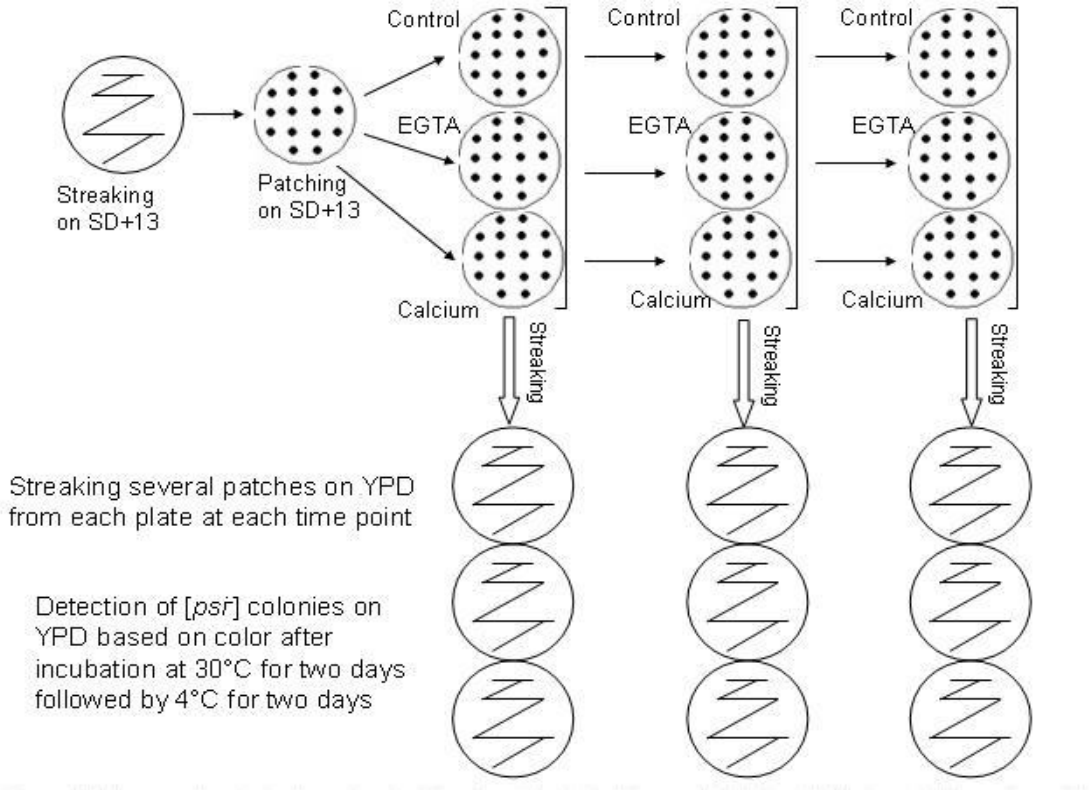

Figure 7.1 **The experimental scheme for studying the effect of calcium on [*PSI*$^+$] instability in *get2Δ* through serial passages on complete synthetic medium**

The level of [*PSI*+] instability is decreased in the presence of 10 mM $CaCl_2$ as compared to control since there are less red or [*psi*$^-$] colonies. On the other hand, 780 µM EGTA which removes calcium, increases [*PSI*$^+$] instability as seen by the appearance of more red colonies (Fig. 7.2 and Table 7.1)

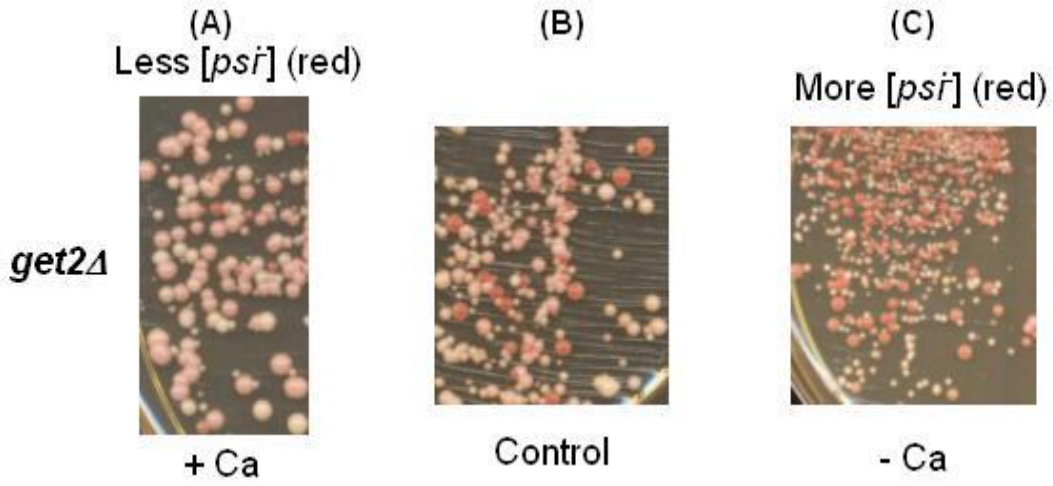

Figure 7.2 **Effect of calcium on [*PSI*$^+$] instability in *get2Δ* through incubation on complete synthetic medium**
Get2Δ strain was incubated for several passages on three different types of synthetic media as shown in figure 7.1A. Colonies were picked at each time point and streaked out on YPD. The level of [*PSI*$^+$] instability is decreased in the presence of 10 mM $CaCl_2$ **(A)** as compared to control **(B)** since there are less red or [*psi*$^-$] colonies. On the other hand, 780 µM EGTA which removes calcium, increases [*PSI*$^+$] instability as seen by the appearance of more red colonies **(C)**. Levels of calcium ions modulate the effects of *get2Δ* on [*PSI*$^+$] propagation. See table 7.1 for tabulated data.

Table 7.1 **Effect of calcium on [*PSI*$^+$] instability in *get2Δ* through incubation on complete synthetic medium**

Strain	**Control**				**+ Ca**				**- Ca**			
	Ade$^+$	Ade$^-$	Total	**% Ade$^-$**	Ade$^+$	Ade$^-$	Total	**% Ade$^-$**	Ade$^+$	Ade$^-$	Total	**% Ade$^-$**
Wild type	100	0	100	0	100	0	100	0	100	0	100	0
get2Δ	50	46	96	47.9	97	9	106	0.7	50	79	129	61

See figure 7.2 for experimental details.

Another version of this experiment was performed where the strains were streaked out on the three different media (control, +Ca and –Ca) and then patched on the

same media. Therefore, the origin of patches on the three plates was different in this case. However, similar effects of calcium and EGTA on [*PSI*$^+$] instability in *get2Δ* strain were observed.

Effect of calcium on [*PSI*$^+$] curing

Also, [*PSI*$^+$] curing experiment was performed in *get2Δ* strain in the presence of calcium to see its effect. Wild type and *get2Δ* strains were transformed with high *HSP104* (pLH105) or control (YEp13) plasmids and the transformants were selected on –Leu medium. The colonies were patched on YPD to analyze the [*PSI*$^+$] curing based on color. The presence of calcium restores some [*PSI*$^+$] curing by excess Hsp104. [*PSI*$^+$] loss can be detected by reddish color on edges in the *get2Δ* strain. Eight transformants were tested and similar result was observed in five. The wild type strain is completely cured by excess Hsp104 as seen by red color. (Fig. 7.3).

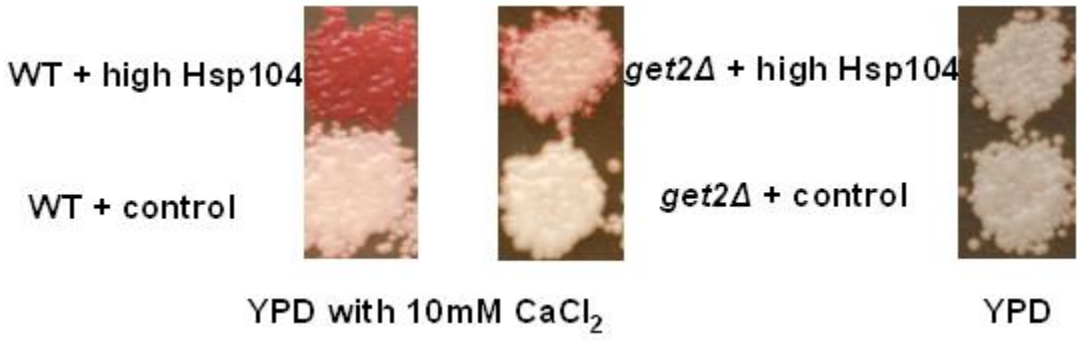

Figure 7.3 **Effect of calcium on [*PSI*$^+$] curing in *get2Δ***
Wild type and *get2Δ* strains were transformed with pLH105 (P_{GPD}-*HSP104)* or control plasmids, patched on -Leu media and incubated for ~4 days. The –Leu plate was replica plated to YPD (with or without 10mM $CaCl_2$) and incubated at 30°C for two days. This was followed by incubation at 4°C for two days. The presence of calcium restores some [*PSI*$^+$] curing by excess Hsp104. [*PSI*$^+$] loss can be detected by reddish color on edges in the *get2Δ* strain. Eight transformants were tested and similar result was observed in five. The wild type strain is completely cured by excess Hsp104 as seen by red color. The controls are shown for comparison. Note that there is no curing in *get2Δ* by excess Hsp104 in the absence of calcium as shown on right.

Conclusion

[*PSI*$^+$] mitotic stability and [*PSI*$^+$] curing by excess Hsp104 are partially restored in the *get2Δ* strain by addition of $CaCl_2$. This shows that some effects of *get2* on [*PSI*$^+$] propagation are expressed due to changes in calcium ion levels.

CHAPTER 8

DISCUSSION

In this study we demonstrate a connection between the Get pathway and yeast prions. *Get2* affects prion maintenance, and *get1*, *get2* and *get3* affect [*PSI*$^+$] curing by excess Hsp104; thus there is a role played by the Get complex in prion maintenance and propagation. The other yeast prion, [*PIN*$^+$], is also affected by the deletion of *get2*. These defects are not due to any decrease in the Hsp104 levels or activity.

However, the range of sizes of [*PSI*$^+$] polymers is wider, and there are more of aggregates of larger sizes in *get2Δ* strain. It is possible that Get2 plays a role in the efficient action of Hsp104 on the [*PSI*$^+$] aggregates. It could do this by presenting the aggregates to Hsp104 chaperone in a manner that allows their effective breakdown into smaller seeds. More specifically, vesicles containing Get complex might directly interact with aggregates and keep them from further growth or agglomeration into larger structures (Fig. 8.1). In the absence of this activity, some aggregates might grow much bigger in size and become unstable. It is known that larger size of aggregates impairs transmission and mitotic stability as reported previously (26, 28). This could explain the prion propagation defect in *get2* mutants.

We may check the hypothesis proposed above by investigating whether Get complex colocalizes with the prion aggregates. We have generated wild type strain OT56 containing *GET2-GFP* and *GET2-HA* fusion constructs. On introduction of a plasmid having a *SUP35-RFP* fusion into the *GET2-GFP* strain, we may utilize fluorescence microscopy techniques to study localization of these two proteins with respect to each other. Based on the model proposed in Fig. 8.1, we expect that Get2 and Sup35 will colocalize with each other.

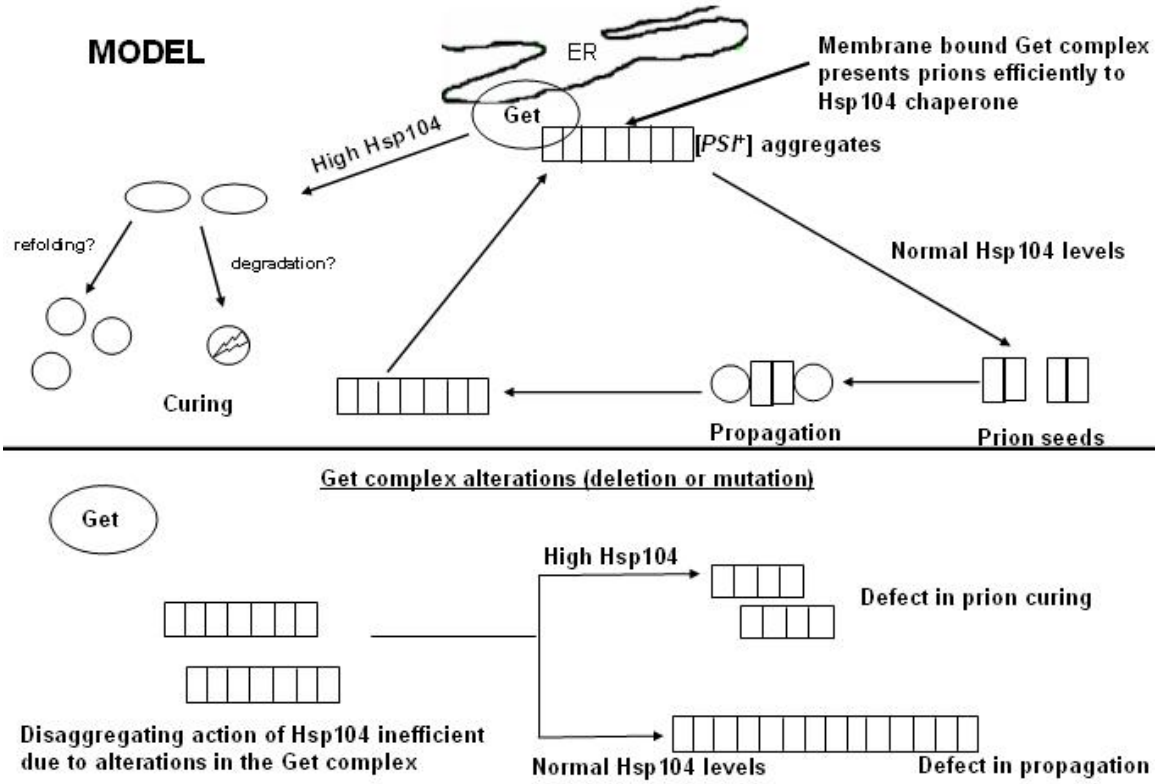

Figure 8.1 **Possible mechanism for the effect of the Get complex on prion propagation**

Also, *get2Δ* might affect the integrity of the Get complex which might lead to perturbations in calcium ion homeostasis. Get1 has been implicated in the regulation of calcium ion fluxes in the ER (29). Also, Get3 is known to have similarities to metal transporters (15). It has been reported previously (30) that metal ions influence aggregation of other prion or amyloid proteins e.g. copper affects PrP aggregation. Also, effects of metal ions on heat shock proteins have been reported (37). We have observed that the levels of calcium ions modulate the effects of *get2Δ* on [*PSI*$^+$] propagation (Figs.7.2 and 7.3 and table 7.1). It is possible that defects in Get complex and/or retrieval of ER proteins may influence the influx of calcium. Calcium may influence Sup35 aggregation or chaperone induction, and in this way counteract the effects of Get complex disruption. Therefore, we see that [*PSI*$^+$] mitotic stability and [*PSI*$^+$] curing by excess Hsp104 are partially restored in the *get2Δ* strain by addition of $CaCl_2$.

We have also observed that prion instability is seen only on synthetic medium. This may suggest that interaction with Get complex becomes important only in certain conditions. On synthetic medium yeast cells become partly stressed and levels of chaperones are higher. We know that some chaperones e.g. Hsp70-Ssa promote prion aggregation (31). This could suggest the importance of the Get complex activity of preventing further growth of aggregates in such conditions. In the absence of this function of the Get complex, the prions become unstable.

The effect of *get2* is not seen in a weak [*PSI*$^+$] background. The dependence of the *get2* effect on the strength of the prion is worth checking further. There could be differences between the weak and strong prion variants. The aggregates of the weak prion variant may not interact with the Get complex effectively. This might lead to their larger size and lower mitotic stability as compared to the aggregates of the strong prion variant in a wild type cell. However, when there are any alterations in the Get complex, the sizes of the already bigger aggregates do not increase any further and thus we do not see the appearance of prion instability on making deletions in the Get complex genes in a weak [*PSI*$^+$] background.

CONCLUSIONS

1) Deletions of *get3* or *get1* exhibit defects in [*PSI*$^+$] curing by excess Hsp104 and deletion of *get3* exhibits a defect in [*PSI*$^+$] maintenance, that are in the same direction as defects caused by *get2* mutants. This shows that some functions that are common for different components of Get complex are involved in prion maintenance.

2) *Get2* deletion causes instability of [*PIN*$^+$] prion on synthetic media in a strong [*PSI*$^+$] background. This shows that effects of Get complex on prions are not specific only to [*PSI*$^+$].

3) *De novo* induction of [*PSI*$^+$] by Sup35 overproduction in the presence of [*PIN*$^+$] is not significantly affected by *get2Δ*.

4) G*et2* deletion does not affect mitotic stability of the weak [*PSI*$^+$] variant.

5) Hsp104 levels are increased rather than decreased in the *get2Δ* and *get2-473* strains, as compared to wild type. This shows that the prion curing and maintenance defects in the *get2* mutants are not due to a decrease in Hsp104 levels.

6) Induced thermotolerance is not affected while basal thermotolerance in synthetic media is increased rather than decreased in the *get2* mutants as compared to wild type. This shows that the prion curing and maintenance defects are not due to a decrease in the activity of Hsp104.

7) The range of sizes of [*PSI*$^+$] polymers is wider, and there are more of aggregates of larger sizes in *get2Δ* strain.

8) [*PSI*$^+$] mitotic stability and [*PSI*$^+$] curing by excess Hsp104 in the *get2Δ* strain are partially restored by addition of $CaCl_2$. This shows that some effects of *get2* on [*PSI*$^+$] propagation are expressed due to changes in calcium ion levels.

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